Identification of Genetic Aberrations in Myelodysplastic Syndromes

Saskia Langemeijer

Identification of genetic aberrations in myelodysplastic syndromes

Idenfication of genetic aberrations in myelodysplastic syndromes

ISBN: 978-90-6464-601-0

Drukwerk: GVO drukkers & vormgevers B.V., Ede
Vormgeving: GVO drukkers & vormgevers B.V., Ede
Omslagontwerp: J. Middelbeek, www.jajem81.nl

Dit onderzoek werd mede gefinancierd door ZonMw (AGIKO stipendium 92003420)

Identification of genetic aberrations in myelodysplastic syndromes

Proefschrift

ter verkrijging van de graad van doctor

aan de Radboud Universiteit Nijmegen

op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,

volgens besluit van het college van decanen

in het openbaar te verdedigen op dinsdag 27 november 2012

om 13.30 uur precies

door

Saskia Maria Carola Langemeijer

geboren op 4 juni 1980

te 's-Hertogenbosch

Promotoren:

Prof. dr. C.G.J. Sweep

Prof. dr. T.J.M. de Witte

Copromotoren:

dr. J.H. Jansen

dr. B.A. van der Reijden

Manuscriptcommissie:

Prof. dr. A.H.M. Geurts van Kessel

Prof. dr. J.H.J.M. van Krieken

Prof. dr. G.J. Ossenkoppele (VU medisch centrum, Amsterdam)

Contents

Chapter 1

Introduction

Parts of this introduction have been published in:

S.M.C. Langemeijer, M.G. Aslanyan, J.H. Jansen. TET proteins in malignant hematopoiesis. Cell Cycle 2009; 8: 4044-4048

S.M.C. Langemeijer, A.O. de Graaf, J.H. Jansen. IAPs as therapeutic targets in hematological malignancies. Exp Opin Ther Targets 2008; 12: 981-993

J.H. Jansen and S.M.C. Langemeijer. The biological basis of MDS. European Hematology Association (EHA) Education Book 2010.

Mature blood cells perform a variety of tasks, involving the transport of oxygen to all tissues by erythrocytes (red blood cells), clotting of blood mediated by thrombocytes (platelets) and the defense against infectious agents by leukocytes (white blood cells). Most of these cells have a life span of only hours to weeks. This implicates that the adult human hematopoietic system must be capable of producing billions of blood cells each day during the life of an individual. In case of an event such as infection or bleeding, the demand for specific blood cells is even greater and production has to increase further. Considering the important tasks of the mature blood cells, an insufficient quantity or quality of these cells, as occurs in myelodysplastic syndromes, may have profound consequences.

Hematopoiesis

Although hematopoietic cells perform a variety of functions, all differentiated blood cells originate from the same hematopoietic stem cell (HSC) population (**Figure 1**)[1-4]. By definition, HSCs have the ability to self-renew and to give rise to progeny that can develop into mature blood cells. In the past, the first step of differentiation was thought to involve the choice between the lymphoid cell lineage (giving rise to T-cells, B-cells and NK-cells) and the myeloid cell lineage (giving rise to erythrocytes, platelets, granulocytes and monocytes). More recently, experiments using mouse models indicated that lymphoid progenitor cells maintain the ability to produce myeloid cell types[5-9]. This phenomenon also seems to apply to adult human hematopoiesis, although the potential of the different progenitors may not be identical to murine blood cell formation[10]. The extent to which "cross-over" between the original myeloid and lymphoid lineages occurs in physiological conditions in the adult human body, is not known and the current models of hematopoiesis will be subject to further adjustments in the future.

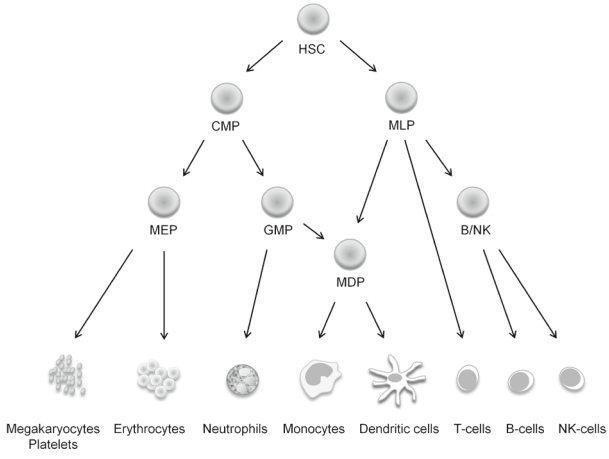


Figure 1
The hematopoietic stem cell (HSC) gives rise to all blood cells. The path of differentiation that the hematopoietic cells follow to become one of the mature blood cell types, is still subject of study. Recent experiments suggested the existence of a multilymphoid progenitor that gives rise to B-cells and NK-cells as well as dendritic cells and macrophages[10]. HSC hematopoietic stem cell; CMP common myeloid progenitor; MLP multilymphoid progenitor; MEP megakaryocyte-erythroid progenitor; GMP granulocyte-monocyte progenitor; MDP monocyte-dendritic cell progenitor.

Although the route from HSC to specialized blood cell is not fully elucidated yet, it is clear that the direction in which the cells develop is influenced by both intrinsic and extrinsic factors. Within the bone marrow a limited number of specialized niches exist in which the HSCs reside. The interaction

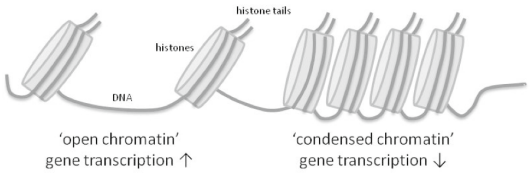
between HSCs and their niche is thought to balance the quiescence and self-renewal of HSCs as well as control their differentiation[11]. Signaling pathways including Notch and Wnt appear to be involved in these interactions[11-16]. In order to guide proliferation and differentiation of HSCs and progenitor cells, hematopoietic growth factors mediate signals via receptors on the surface of these cells[17]. Some growth factors act at early stages of hematopoiesis and stimulate differentiation towards multiple cell types, whereas others stimulate a specific cell lineage. Examples of the first group are Stem Cell Factor (SCF) and IL-3, which stimulate the proliferation of HSCs and progenitor cells. Examples of more lineage-specific growth factors are erythropoietin (Epo), which promotes the proliferation of erythroid progenitor cells, and granulocyte colony stimulating factor (G-CSF), which stimulates the production of neutrophils. After binding of growth factors to their receptors, signals are passed by activation of kinases, for example of the Janus kinase family (JAKs), initiating a cascade of secondary events that eventually lead to changes in cell survival, proliferation and differentiation. In addition to these extracellular signals, hematopoietic cell fate is influenced by a network of transcription factors. As the cells differentiate, specific transcription factors must be expressed at a certain level, whereas other transcription factors must be switched off. Changes in the balance between transcription factors can change the fate of the cell. For example, *Pax5* drives differentiation towards B-cells at the expense of other cell lineages[18;19]. The transcription factor *PU.1* activates myeloid- or B-cell specific gene expression, depending on the presence of other transcription factors, while genes associated with erythroid, NK- or T-cell development are repressed[20-23]. *C/EBPα* suppresses both lymphoid and erythroid development, favoring myeloid differentiation[24]. Considering the important influence that transcription factors exert on cell development, their expression must be tightly regulated. Epigenetic changes are thought to play an important part in this regulation and have recently been the subject of many studies. Epigenetics is defined as the study of heritable changes in gene activity that cannot be explained by changes in the DNA sequence itself. Epigenetic regulation includes processes such as DNA methylation and histone tail modifications (**Box 1**). Although these modifications can be preserved during cell division, the epigenetic marks may also change. The genomic regions in which histone tail modifications and DNA methylation are present, the combined presence of various modifications and the extent of modification (e.g. di- or trimethylation of lysines) influence the expression of HSC and cell lineage associated genes and thereby play a role in directing cell fate. It has been suggested that HSCs contain a permissive chromatin structure, in which repressive and activating modifications may occur alone or together near the same genes and additional cofactors must be present to induce an effect on gene transcription. This epigenetic plasticity may be progressively reduced in differentiating cells[25-28].

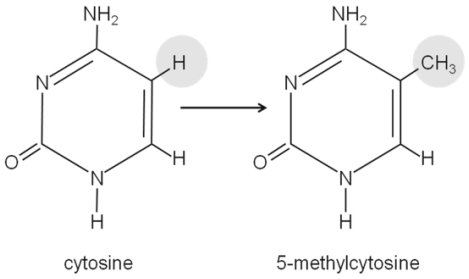
Hematopoietic malignancies

If a defect in the HSC or an early hematopoietic progenitor cell (HPC) arises that disturbs the finely balanced process of hematopoiesis mentioned above, this cell may obtain a proliferative advantage compared to the other HSCs or HPCs. This results in a clonal (malignant) hematopoiesis. The

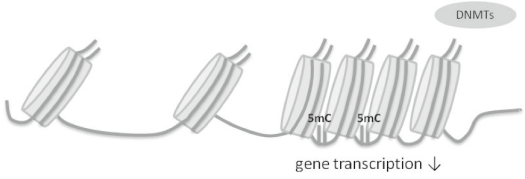
Box 1

DNA is the same in all somatic cells of a human being (although exceptions exist; consider DNA rearrangement in lymphocytes). However, these somatic cells perform a variety of functions. This can be explained by changes in the activity of specific sections of DNA. The changes in DNA transcription through modulation of chromatin is defined as epigenetics.

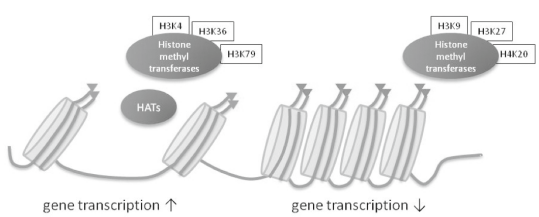




Chromatin can be modified in a number of ways. Two important ways are methylation of DNA and modification of histones. DNA methylation of cytosines within gene regulatory DNA sequences can influence the transcription of the related gene(s). It is associated with a closed and less accessible configuration of the chromatin. In general, repression of the associated gene occurs either directly or through concomitant epigenetic events, such as recruitment of repressive protein complexes. DNA methylation is catalyzed by the family of DNA methyltransferases (DNMTs). The methylation patterns can be maintained when cells divide, although changes may occur.



Histone tail modifications include phosphorylation, acetylation, methylation, sumoylation and ubiquitylation. These modifications can lead either to activation or repression of the associated genes. Acetylation and methylation of histones were among the first covalent modifications to be described. Acetylation occurs mainly at lysine residues in the amino termini of histones H3 and H4 and leads to activation. Acetylation is mediated by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs). Methylation of histone H3 at certain positions (K4, K36, K79) results in activation, whereas this process leads to gene repression when other loci are affected (H3K9, H3K27, H4K20). Histone methyltransferases are responsible for these modifications and include e.g. MLL proteins (H3K4 methylation) and EZH2 (H3K27 methylation). Multiple histone demethylases have been described, such as the JmjC (jumonji)-containing family. These epigenetic changes to DNA and histones appear to be interrelated, for example, after DNA methylation occurs, MeCP2 (methyl-CpG binding protein 2) can be recruited that attracts HDACs to reverse the activating histone acetylation.



characteristics of the malignancy that arises depends on the stage of hematopoiesis in which the defect occurs and its potential to influence differentiation into specific lineages. Hematopoietic malignancies are grouped into myeloid and lymphoid diseases. Since the research described in this thesis mainly focuses on myeloid malignancies, in particular myelodysplastic syndromes, these will be discussed here. According to the latest World Health Organization (WHO) classification, myeloid malignancies are subdivided into myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS), an intermediate entity with features of both MPN and MDS (MPN/MDS), acute myeloid leukemia (AML) and myeloid/lymphoid neoplasms with eosinophilia and defects of the *PDGFRA* , *PDGFRB* or *FGFR* genes[29]. The prominent morphological feature of MPN is proliferation of one or more of the myeloid lineages. The bone marrow is hypercellular and patients have increased numbers of mature hematopoietic cells in their blood. The majority of MPN have now been associated with specific aberrations that involve genes encoding protein kinases, particularly *JAK2*, that transmit extracellular signals leading to stimulation of proliferation. Like MPN, myelodysplastic syndromes are characterized by increased proliferation as well. However, increased cell death of the defective cells occurs simultaneously, leading to an ineffective hematopoiesis with cytopenias in the peripheral blood. Moreover, cells show dysplastic morphological features. Although multiple large chromosomal aberrations have been found in the DNA of bone marrow cells from MDS patients, until recently, defects of specific genes have been described in only a minority of patients, which will be discussed later. This is in sharp contrast to AML, where a variety of genes have been found to be affected in a substantial number of patients. In AML differentiation of hematopoietic cells along one or more lineages is completely blocked at a certain stage of differentiation.

Myelodysplastic Syndromes

The term myelodysplastic syndromes refers to a wide variety of malignant hematopoietic disorders. Dysplastic features of the erythroid, granulocytic, monocytic or megakaryocytic blood cell lineages is one of the main characteristics of these diseases, however they are not specific for MDS. Due to the lack of a clear common denominator, a variety of names has been used to describe (a subtype of) MDS. “Refractory anemia”, for example, referred to the fact that patients with MDS did not respond to the therapies commonly used to treat anemia, and the names “smoldering leukemia” or “preleukemia” were used because some patients with MDS eventually developed acute myeloid leukemia. The French-American-British (FAB) classification, published in 1976 and revised in 1982, was designed to help define MDS based on morphological examination of the blood and bone marrow (**Table 1**)[30;31]. The clinical phenotype of patients with refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS) was found to be more chronic and less aggressive than that of patients of the other groups, whose chance of developing AML was higher and survival worse[32].

Table 1

French-American British (FAB) Classification of myelodysplastic syndromes (1982)[30;31]
based on:
percentage of blasts in blood (<1%, 1-5%, >5%) and bone marrow (<5%, 5-20%, 20-30%)
presence or absence of ring sideroblasts
presence or absence of Auer rods
number of (pro)monocytes (above or below $1.0x10^9$/l)
categories:
refractory anemia (RA)
refractory anemia with ring sideroblasts (RARS)
refractory anemia with excess of blasts (RAEB)
refractory anemia with excess of blasts in transformation (RAEB-t)
chronic myelomonocytic leukemia (CMML)

Thus, this classification provided tools to diagnose MDS and predict its clinical course and has been used for many years. However, several pitfalls remained. The FAB classification did not take into account the number of cell lineages affected by cytopenias and/or dysplasia. In addition, the FAB defined entity of chronic myelomonocytic leukemia (CMML) was shown to possess features that were characteristic of myeloproliferative disorders as well as MDS[33;34]. RAEB-t , the most aggressive MDS subtype, was defined by an increased percentage of blasts in the blood (>5%) or bone marrow (20-30%) or the presence of Auer rods. The importance of this category was questioned later, since the prognostic impact of the presence of Auer rods was less important than previously thought[35]. Moreover, the clinical relevance of distinguishing patients with dysplasia and 20-30% blasts (RAEBt) or more than 30% blasts (AML with dysplasia) seemed to be limited. The presence of cytogenetic aberrations was not included in the FAB classification. To attempt to improve the existing methods for risk assessment in MDS, the International Prognostic Scoring System (IPSS) was developed in 1997 (**Table 2**)[36]. In this system, the percentage of blasts, the number of cytopenias and the presence of cytogenetic abnormalities was taken into account, leading to a risk score correlating with the prognosis of the patient. The IPSS score was often used in combination with the FAB classification. In 2001 the WHO proposed modified criteria to distinguish MDS subtypes which should replace the FAB classification (**Table 3**)[37-39]. The WHO classification redefines CMML as a myeloproliferatieve/myelodysplastic neoplasm (MPN/MDS) and MDS with 20-30% blasts in the bone marrow as AML. It recognizes dysplastic changes in the granulocytic and megakaryocytic lineages in addition to dysplastic changes of the erythroid cell line and defines the group of refractory anemia with multilineage dysplasia (with or without ringed sideroblasts) in addition to the group of refractory anemia. Cases with anemia, low blast counts and an isolated deletion of the long arm of chromosome 5 were categorized as a separate entity. Although this classification more precisely distinguished MDS subtypes, several difficulties remained. For example, the group of patients with a cytopenia of one cell lineage but multilineage dysplasia cannot be clearly categorized. Other ways to classify these patients have been proposed[40]**.** In 2008, the WHO classification was revised and a new subgroup named refractory cytopenia(s) with unilineage dysplasia (RCUD, comprising

refractory anemia, refractory neutropenia and refractory thrombocytopenia) was added[29]. Although this revision does not solve all problems in classification, it further refines the MDS subtypes. Several newer risk-scoring systems that take additional parameters into account have been published or are being developed at this moment[41-44]. For example, the WHO classification-based prognostic scoring system (WPSS)[41] takes into account karyotypic abnormalities and transfusion dependency, and can be used to estimate survival and risk of progression towards AML at any time during the course of the disease.

Table 2

International Prognostic Scoring System (IPSS) (1997)[36]
based on:
percentage of blasts in the bone marrow (<5%, 5-10%, 11-19%, 20-30%)
number of cytopenias (0-1 versus 2-3)
cytogenetic abnormalities
- good: no abnormalities, -Y, del(5q), del(20q)
- poor: 3 or more abnormalities, chr 7 abnormalities
- intermediate: other abnormalities
categories:
low-risk
intermediate-1 risk
intermediate-2 risk
high risk

Table 3

World Health Organization (WHO) classification of myelodysplastic syndromes (2001)[37;38]
based on:
percentage of blasts in blood (<1%, 1-5%, >5%) and bone marrow (<5%, 5-9%, 10-19%)
number of cytopenias (anemia versus bicytopenia or pancytopenia)
number of cell lineages affected by dysplasia (erythroid dysplasia versus multilineage dysplasia)
presence or absence of ring sideroblasts
presence or absence of Auer rods
presence of an isolated del(5q)
categories:
refractory anemia (RA)
refractory anemia with ring sideroblasts (RARS)
refractory anemia with multilineage dysplasia (RCMD)
refractory anemia withmultilineage dysplasia and ring sideroblasts (RCMD-RS)
refractory anemia with excess of blasts (RAEB-1 and RAEB-2, depending on percentage of blasts)
myelodysplastic syndrome unclassified (MDS-U)
myelodysplastic syndrome with isolated del (5q)

Pathogenesis of myelodysplastic syndromes

Diagnosing MDS and subdividing patients into prognostically relevant groups would be facilitated by thorough knowledge of the origin of these diseases. The pathogenesis of MDS is complex and may differ depending on the MDS subtype. Several factors that may influence MDS development have been studied, including disorders in the regulation of programmed cell death and immune responses and (epi) genetic abnormalities.

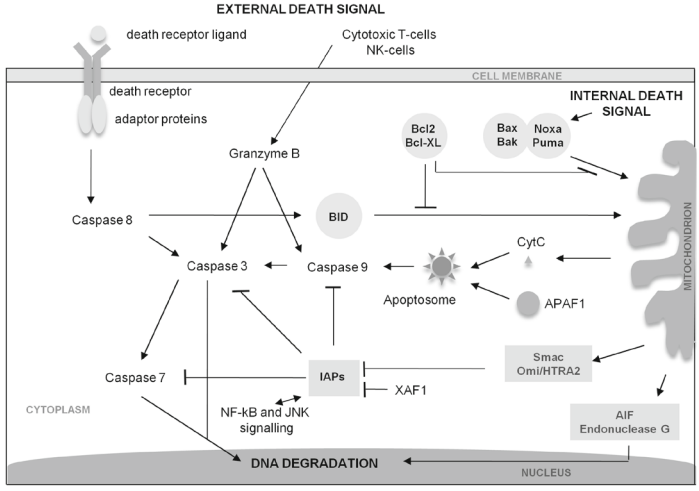
Deregulation of programmed cell death

One of the hallmarks of MDS is ineffective hematopoiesis resulting in peripheral cytopenias. Increased cell death has been hypothesized to contribute to the failure to produce sufficient mature blood cells. During the transformation of MDS to AML, cell death in the immature bone marrow cells may be reduced, causing a proliferative advantage of these cells. Different forms of cell death have been distinguished[45;46]; apoptosis, autophagy-associated cell death and necrosis. Apoptosis is an ordered process of cellular destruction which is executed by specific enzymes (caspases) that are present in cells in an inactive form, but become activated when cells are at the end of their normal life span, when they are damaged or when they encounter various types of stress, including chemical exposure, viral infection or irradiation **(Box 2)**. The main morphological feature of apoptosis is shrinkage of the cell and condensation and fragmentation of DNA in its nucleus. Apoptosis can be triggered by intracellular signals, such as DNA damage, and extracellular signals, such as those mediated via the death receptors on the cell membrane. Autophagy is a process which targets cellular proteins and organelles for degradation in lysosomes. Autophagy is a protective mechanism to allow cell survival during the initial stages of starvation and other forms of stress by maintaining production of ATP (energy) and eliminating damaged proteins and organelles that accumulate during stress. The role of autophagy in cell death is controversial. It is not clear if autophagy can continue to the point of caspase-independent cell death without signs of apoptosis. Some genes that have been implicated in regulation of apoptosis, such as *BCL2* and *TP53*, have been linked to regulation of autophagy as well[45]. The third type of cell death is necrosis. In general, necrosis is regarded as accidental (not programmed) cell death. More recently it has been suggested that regulated forms of necrosis exist, mediated by, for example, TNFα. The various forms of cell death can be studied using techniques such as electron microscopy (all types of cell death), TUNEL/ISEL assays (apoptosis) and determination of expression of cell death-associated proteins (e.g. caspases in apoptosis). In most studies, the observation of cytopenias in MDS despite an often hypercellular bone marrow has been attributed to defects in the regulation of apoptosis. On examination of the bone marrow of MDS patients, increases in apoptotic markers have been described[47-54]. Several studies indicated that apoptosis was increased in particular in the indolent forms of MDS, whereas the aggressive phenotype was characterized by lower levels of apoptotic markers. However, conflicting results have been published. The methods used to determine the level of apoptosis, the cell lineages studied and the selection of patients may have led to these controversies. Deregulation of expression of specific proteins that are involved in the execution of apoptosis have been described. For example,

it has been reported that expression of *BCL2* is decreased in the smoldering MDS cases and/or increased in the aggressive phenotype compared to the expression in normal bone marrow[52;55-58]. However, as mentioned above, *BCL2* has been implicated in autophagy as well[59]. Signs of increased autophagy, such as double-membraned autophagic vacuoles, have been found in bone marrow of patients suffering from MDS[60]. Thus, deregulation of programmed cell death seems to play a role in the development of MDS. Stimulation of proliferation of the hematopoietic cells with growth factors such as Epo, G-CSF or Thrombopoietin (TPO) can limit the detrimental effects of hematopoietic cell death in some patients.

Box 2

Apoptosis or programmed cell death of the mammalian cell is regulated by specialized enzymes, particularly caspases. Caspases are present in the cell in an inactive form and can be activated via an extrinsic or intrinsic route (Figure). The initiator caspases (most importantly 8 and 9) translate an intra- or extracellular death signal into proteolytic activity targeting effector caspases. Effector caspases (most importantly 3 and 7) in turn are responsible for the subsequent proteolytic events that result in destruction of the cell. Under normal circumstances, apoptosis is initiated when the cell receives either an extracellular or intracellular death signal. Examples of extracellular death signals are binding of TNFα to its receptor and exposure of the cell to granzyme B produced by cytotoxic T-cells or NK-cells. Activation of a death receptor leads to cleavage of initiator caspase 8 and, subsequently, effector caspases 3 and 7. Granzyme B activates caspases 3 and 9 in the cytoplasm. An important intracellular death signal is the presence of DNA damage. Upon this signal, the mitochondrial membrane potential is altered, resulting in the release of cytochrome-c and apoptotic peptidase activator 1 (APAF1). These proteins recruit initiator caspase 9 to form a protein complex called the apoptosome. The apoptosome can induce effector caspase 3, which subsequently leads to cleavage of caspase 7.



Deregulation of immune responses

T-cells are in general not regarded as part of the malignant clone in MDS[61-64]. However they may affect the development of the disease. Apoptosis can be induced by a T-cell mediated attack on the bone marrow cells of MDS patients. Increased production of cytokines and an increased expression of death-signal mediating receptors, such as Fas, on hematopoietic cells have been found in MDS[65-68]. After recognition of antigens on the outer membrane of target cells, proliferation of T-cells can result in clonal or oligoclonal T-cell expansions. Antigens may consist of novel peptides that are not expressed in healthy cells. Alternatively, the expression of known antigens may be increased, eliciting an immune response. Although skewing of the T-cell receptor repertoire has been shown to occur in the healthy elderly population as well, it is increased in MDS patients independent of their age, in particular in the CD8+ cells[69-72]. Activated cytotoxic T-cell subsets also seem to be increased in MDS compared to healthy controls[63;71;73]. In addition, a high number of T-helper 17 cells are present in low-risk MDS [74]. Th17 cells are thought to induce the production of inflammatory cytokines and increased numbers may contribute to the auto-immune response seen in MDS. Alternatively, the response elicited by these Th17 cells and also cytotoxic T cells may prevent further disease progression. A subset of MDS patients are responsive to treatment with anti-thymocyte-globuline (ATG) and/or cyclosporine[75-79]. ATG has been shown to induce loss of oligoclonally expanded T-cell populations that are responsible for suppression of bone marrow cells[80;81]. In addition to the changes in cytotoxic and helper T cells, increased amounts of $CD4^{+}CD25^{high}Foxp3^{+}$ regulatory T-cells (T_{reg}) have been found in patients with more aggressive forms of MDS, suggesting that T_{reg}-mediated immune suppression during these disease stages may allow continuing expansion of the malignant cells[82;83]. Furthermore, disease progression may occur due to expression of proteins on the MDS cell membrane that suppress activated T-cells. For example, B7-H1 on the MDS cell membrane has been suggested to interact with PD1 on the activated T-cells[84]. Not only T-cells, but also other cells of the immune system have been associated with MDS. For example, NK-cell function in MDS patients is altered [85;86]. In conclusion, the number and activation status of various immune cells may influence the development and course of MDS, not only by induction of apoptosis but also by other mechanisms.

Epigenetic abnormalities in MDS

Epigenetic changes are increasingly recognized as important mechanisms by which gene transcription is regulated. Aberrant DNA methylation of genes involved in cell-cycle regulation, apoptosis and differentiation has been shown In various types of cancer. In MDS, hypermethylation of a number of genes has been reported (**Table 4**). Hypermethylation of some of these genes has been shown to correlate with a poor prognosis and worse response to therapy[87;88]. One of the most studied genes is *CDKN2B* (p15INK4B), a cyclin-dependent kinase (CDK) inhibitor that controls cell cycle progression. Silencing of this gene by methylation may contribute to uncontrolled cell growth. *CDKN2B* is frequently methylated in patients with high-risk MDS compared to low-risk patients[89;90]. In patients without *CDKN2B* methylation, methylation was often acquired at the time of progression to leukemia. More recently, global methylation patterns in MDS patients have been the focus of several studies [91-93]. The results show that hypermethylation in MDS is not restricted to a

small subset of genes. Römermann et al. showed that an increase in global methylation could already be found in low-risk MDS patients and it was further increased in the high-risk patient group[91].

Table 4

Hypermethylated genes in MDS			
Gene		Location	References
CALCA	Calcitonin	11p15.2	155;156
CDH1	E-cadherin	16q22.1	88;157-159
CDH13	H-cadherin	16q23.3	88
CDKN2B (p15)	Cyclin-dependent kinase inhibitor 2B	9p21	89;90;157;159-168
CTNNA1	Catenin	5q31	110;169
DAPK	Death-associated protein kinase	9q34.1	158;159;170;171
DDIT3	DNA-damage-inducible transcript 3	12q13.1-13.2	172
ER	Estrogen receptor	6q25.1	88;157
FHIT	Fragile histidine triad gene	3p14.2	173;174
GRAF	Rho GTPase activating protein 26	5q31	175;176
HIC1	Hypermethylated in cancer 1	17p13.3	87;157
ID4	Inhibitor of DNA binding 4	6p22.3	177
KLF11	Kruppel-like factor 11	2p25	178
MEG3	maternally expressed 3	14q32	179
NOR1	Organic solute carrier partner 1	1p34.3	88
NPM2	Nucleophosmin 2	8p21.3	88
OLIG2	Oligodendrocyte lineage transcription factor 2	21q22.11	88
PGR	progesterone receptor	11q22	88
RASSFIA	Ras association (RalGDS/AF-6) domain family member 1	3p21.3	180
RB1	Retinoblastoma 1	13q14.2	167
RIL	Reversion-induced LIM protein	5q31.1	88;181
SNRPN	small nuclear ribonucleoprotein polypeptide N	15q11.2	179
SOCS1	Suppressor of cytokine signaling 1	16p13.13	159;180;182;183

Subsequent studies confirmed the aberrant methylation of promoter regions in high risk MDS patients and provided large numbers of differentially methylated genes in MDS patients compared to healthy controls[92;93]. In healthy cells DNA methylation is required for maintaining silence in nonexpressed and noncoding regions of the genome. The cause of the aberrant methylation patterns in MDS is not clear. As mentioned before, DNA methylation is regulated at several levels, for example by DNA methyltransferases (DNMTs, **box1**). Overexpression of DNMT1 and DNMT3A/B has been shown in bone marrow biopsies of MDS patients[94]. However, expression of DNMTs was not aberrant when isolated hematopoietic cells were studied, although correlations between DNMT subtypes and promoter methylation of specific genes in MDS were found[95]. Recently, mutations in DNMT3A have been described in MDS, but the functional consequences of these mutations are not yet clear[96;97]. Although DNA methylation is associated with gene silencing, methylation itself is not sufficient to repress transcription. Other epigenetic changes, such as histone modifications are involved as well (**box 1**). Studies focusing on changes in the histone code in MDS have not been

performed yet. Changes in specific proteins directly or indirectly involved in histone modifications have been reported. For example, defects involving the MLL gene occasionally occur in MDS patients. MLL is a known chromatin modifier and has been implicated in H3K4 methylation among other activities. In addition, in a small minority of patients with MDS a chromosomal translocation involving the *EVI-1* gene occurs. Aberrant expression of EVI-1 in MDS has been detected as well. The mechanisms by which EVI-1 contributes to MDS pathogenesis have not been fully elucidated yet. Recently it was shown that EVI-1 interacts with the histone methyltransferase SUV39H1. SUV39H1 catalyzes the addition of trimethyl groups to lysine 9 of histone 3 (H3K9me3) [98;99]. In this way, aberrations of EVI-1 could influence the histone code. Similar direct or indirect mechanisms that result in epigenetic modifications may apply to other MDS cases. The discovery of aberrations in DNA methylation provided the basis for the application of pharmaceutical compounds which modify this process. DNMT inhibitors replace normal nucleotides in the DNA (and RNA) and bind DNMTs, which leads to degradation of the latter, thus preventing DNA methylation by these proteins during subsequent cell divisions. As mentioned previously, DNA methylation is also dependent on changes in histone modifications. HDACs cause histone deacetylation leading to a repressive state of the chromatin, as does DNA methylation. HDAC inhibitors counteract this mechanism. Treatment with DNMT inhibitors has shown overall response rates (hematological improvement, partial/complete response) in 20-60% of MDS patients (reviewed in [100]). HDAC inhibitors as monotherapy have shown lower response rates. Combinations of both compounds (and of these compounds with other drugs used in MDS) are being evaluated as well.

Genetic abnormalities in MDS

As mentioned before, MDS is not characterized by one or several well-defined genetic defects, as is the case for chronic myelogenous leukemia. In fact, the genetic background of MDS appears highly heterogeneous, as is its clinical phenotype. Karyotype analysis is routinely used in MDS diagnostics and has revealed a multitude of abnormalities. These include deletions, duplications, inversions and translocations, of which deletions are clearly the most common abnormalities, leading to the hypothesis that loss of tumor suppressor genes is an important mechanism in MDS pathogenesis.

At diagnosis, karyotype analysis of hematopoietic cells reveals (a) cytogenetic defect(s) in approximately 50% of patients (**Figure 2**). The number of abnormalities per patient varies and correlates with prognosis[101]. Deletions of (a part of) chromosomes 5, 7 and 20 and trisomy of chromosome 8 occur most frequently (**Figure 3**)[101]. In line with the hypothesis that deletions may result in loss of a tumor suppressor gene, many studies have focused on finding the targeted gene(s) on chromosomes 5, 7 and 20. In order to find genes of interest, the chromosomal areas affected by these deletions have been compared between patients, defining commonly deleted regions (CDRs). On chromosome 5, two distinct CDRs have been described. The most distal region lies on 5q33.1 and contains approximately 40 genes and 3 microRNAs[102]. This CDR was defined by analyzing patients with the 5q-syndrome, characterized by anemia and an isolated del(5q). The proximal CDR is located on 5q31 and has been associated with both MDS and AML that progressed from MDS[103-106]. Recently, the gene encoding ribosomal S14 protein (*RPS14*) on 5q33.1 was implicated

Figure 2[101]
Number of cytogenetic abnormalities in MDS patients (karyotype analysis)

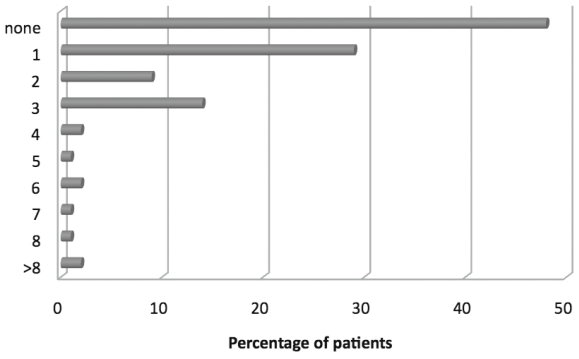
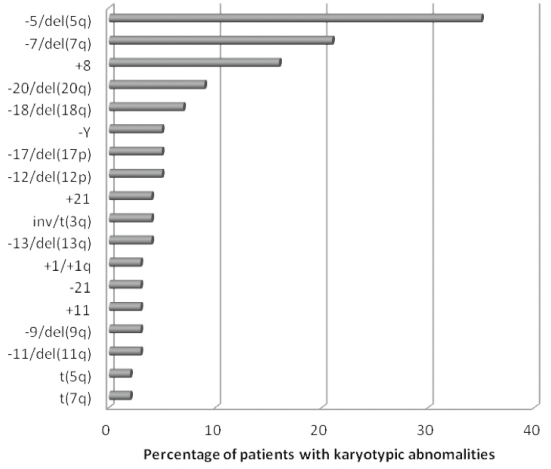


Figure 3[101]
Distribution of most common cytogenetic abnormalities in MDS patients



in the pathogenesis of the 5q- syndrome. Knockdown of this gene with approximately 60% was shown to cause a block in erythroid differentiation of cultured human hematopoietic cells, a phenotype that could be rescued by re-introduction of the gene[107]. In addition, overexpression of *RPS14* in cells of patients with 5q-syndrome promoted erythroid differentiation. The remaining *RPS14* gene in 5q-syndrome patients did not appear to be affected by mutations, microdeletions or hypermethylation[107]. Mice with a deletion of part of the 5q33.1 CDR in hematopoietic cells, containing *RPS14* among other genes, show features of 5q-syndrome[108]. This effect appears to be at least in part mediated by activation of the tumor suppressor protein p53. Loss of RPS14 may not however explain all features of the 5q-syndrome and it is likely that other genes or microRNAs are involved as well. Indeed, miR-145 and miR-146, the first located in the 5q33.1 CDR, have been implicated in the thrombocytosis, neutropenia and megakaryocytic dysplasia that can be seen in 5q-syndrome[109]. The second CDR on 5q31 carries several genes that have been implicated in MDS pathogenesis, including *CTNNA1*[110], *HSPA9*[111;112] and *EGR1*[113]. The alpha catenin CTNNA1 was found to be expressed at much lower levels in primitive hematopoietic cells of individuals with a 5q deletion than in other MDS patients or healthy controls. CTNNA1 expression by the remaining allele in the HL-60 cell line that carries a 5q deletion was reported to be suppressed by both promoter methylation and histone deactetylation[110]. A mutated ortholog of HSPA9 in zebrafish was shown to cause defects in hematopoiesis including apoptosis and dysplasia[112]. EGR1 deficiency in mice caused a myeloproliferatieve disorder with features of myelodysplasia after treatment with N-ethyl nitrosurea to induce secondary genetic defects[113]. These results indicate that multiple genes on 5q31 are probably responsible for the MDS phenotype. Several other genes on chromosome 5q that are located outside the CDRs, but still are affected in multiple patients, have also been implicated in MDS development. Genes located within CDRs on the other commonly deleted chromosomes, 7 and 20, have not been similarly implicated in the pathogenesis in MDS. Several

CDRs on chromosome 7 have been described, including cytobands 7q22, 7q31-33 and 7q36[114-120]. In many patients multiple CDRs are affected, suggesting that the combined effect of several critically deleted genes is important. On chromosome 20, a CDR has been reported on 20q11-20q13 [121-126]. As is the case for chromosome 7, the genes located on chromosome 20 that are responsible for (part of) the MDS phenotype have not been identified yet.

Although large chromosomal aberrations do not directly provide insight into the pathogenesis of MDS, the detection of these defects has improved MDS diagnostics since they provide evidence of clonal disease. In addition, their presence influences prognosis (**Table 5**) and can predict response to therapy, for example in case of patients with 5q- syndrome, who respond well to treatment with lenalidomide[127]. Unlike these large chromosomal aberrations, balanced translocations can more easily provide specific information concerning the affected genes. However, various chromosomal translocations in MDS have been found in single or only a few cases, mostly therapy-related MDS, and are often not the primary cytogenetic defect. Several genes that are recurrently found to be involved in translocations are listed in **Table 6**. Recently, a novel gene, *IER3* was implicated in MDS pathogenesis after it was found to be involved in a translocation between chromosomes 6 and 9. Expression of *IER3*, located on 6p21 was lost due to the translocation. *IER3* is involved in the regulation of apoptosis. Its expression was subsequently shown to be more than four-fold increased or decreased in more than half of MDS patients without 6p21 abnormalities compared to healthy controls[128].

Table 5

Prognostic impact of cytogenetic aberrations in MDS[184]				
Risk Group	Good	Intermediate 1	Intermediate 2	Poor
Percentage of patients	73%	6%	9%	12%
Median survival with supportive care	55 months	29 months	15 months	8 months
Cytogenetics	normal karyotype	+8	-7	> 3 abnormalities
	del(5q)	del(11q)	3 abnormalities	t(5q)
	-Y		any 3q abnormality	
	del(20q)		del(7q)	
	+21		t(11q23)	
	del (12p)		+19	
	t(1q)			
	t(7q)			
	del(9q)			
	t(15q)			
	-X			
	-21			
	t(17q)			
	del(15q)			

Table 6

Genes recurrently involved in translocations in MDS				
Gene		Function	Examples of translocations	References
ETV6	Ets variant 6	Transcription factor	t(10;12)(q24;p13), t(3;12)(q26;p13),	185;186
MDS1-EVI1 (MECOM)	Myelodysplasia syndrome 1-ectopic virus integration site 1	Transcription factor	t(2;3)(p21;q26), t(3;12)(q26;p13)	186;187
RUNX1	Runt-related transcription factor 1	Transcription factor	t(16;21)(q24;q22), t(3;21)(q26;q22)	188;189
MLL	Mixed lineage leukemia	Methyltransferase	t(11;16)(q23;p13.3), t(11;17)(q23;q25)	190;191
PRDM16	PR-domain containing 16	Transcription factor	t(1;21)(p36;q22), t(1;3)(p36;p21)	192;193
NUP98	Nucleoporin 98kD	Nucleocytoplasmic transport	t(11;20)(p15;q11), t(7;11)(p15;p15)	194;195

As has been discussed in the previous section, (epi)genetic defects such as disturbed methylation patterns and chromosomal deletions and duplications may contribute to MDS pathogenesis. However, they often affect many genes, complicating the search for those that are critically involved in this process. Aberrations of specific genes, such as the *JAK2* mutations in MPN, have been found in MDS, although they occur in a minority of cases (**Table 7**). Mutations in the RAS family of genes (*N-RAS, K-RAS, H-RAS*) were among the first recurring mutations identified in MDS[129;130]. RAS proteins are involved in regulation of signaling pathways that are implicated in cell proliferation and survival. In healthy cells, RAS proteins are activated after binding of growth factors or cytokines to plasma membrane receptors with tyrosine kinase activity. In case of RAS mutations, the signaling cascade is constitutively activated, leading to uncontrolled cell growth. Alternatively, mutations of the receptors on the plasma membrane, such as *FLT3*, *CSF1R* and *KIT* may have similar effects. *RAS* and *FLT3* mutations in MDS are associated with a higher risk of disease progression[131-133]. Also, *RAS* mutations are more common in the group of chronic myelomonocytic leukemias (CMML), which are now classified as MPN/MDS. The detection of recurrent RAS mutations in MDS has lead to the application of farnesyl transferase inhibitors. The specific inhibition of the enzyme farnesyl transferase by these drugs interferes with the transfer of a farnesyl moiety to substrates such as RAS proteins. This modification is required in the normal processing of RAS proteins. Clinical responses to these drugs have been seen in MDS, though do not seem to be related to *RAS* mutation status, indicating additional mechanisms of action (reviewed in [134]). Clinical trials in high-risk MDS patients with inhibitors directed against *FLT3* are ongoing. Mutations in other genes involved in signal transduction, such as *PTPN11* and *JAK2,* have been described but are rare in MDS. In contrast, *JAK2* mutations occur frequently (~50%) in refractory anemia with ring sideroblasts and thrombocytosis (RARS-T)[135;136]. RARS-T is now classified as MPN/MDS. Aberrations of genes that are not (directly) involved in signal transduction have been studied as well. *TP53*, a tumor suppressor gene that is known to be mutated in a wide variety of malignancies, has been studied elaborately in MDS. Mutations in *TP53* are found in about 5-15% of patients and are associated with a worse clinical outcome[137].

Table 7

Genes mutated in MDS					
Gene		Location	Gene function	Occurence of mutations	References
H/K/N RAS	Harvey rat sarcoma viral oncogene homolog Kirsten rat sarcoma viral oncogene homolog Neuroblastoma RAS viral (v-ras) oncogene homolog	11p15.5 12p12.1 1p13.2	Signal transduction	5-20% (higher in advanced MDS)	138; 196-201 202;203 204;205 206
FLT3	Fms-related tyrosine kinase 3	13q12	Signal transduction	<1-4%	138;207 208-211
PTPN11	Protein tyrosine phosphatase, non-receptor type 11	12q24	Signal transduction	<1-2	212-214
JAK2	Janus kinase 2	9p24	Signal transduction	<1-5%	138;215;216
CSF1R (FMS)	Colony stimulating factor 1 receptor	5q32	Growth factor receptor	7-8%	204;217
KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	4q11-12	Growth factor receptor	<1-5%	211;218;219
TP53	Tumor protein p53	17p13.1	Apoptosis, DNA repair, cell cycle regulation	5-15%	204;220-225
CDKN2A	Cyclin-dependent kinase inhibitor 2A	9p21	Cell cycle regulation	<1%	226-228
RUNX1	Runt-related transcription factor 1	21q22.3	Transcription factor	3-8%	138;139;229
CEBPalpha	CCAAT/enhancer binding protein (C/EBP), alpha	19q13.1	Transcription factor	<1-4%	230;231
WT1	Wilms tumor 1	11p13	Transcription factor	<1% (AML post MDS)	138;232
ASXL1	Additional sex combs like 1	20q11.1	Chromatin modification	10-20%	138;144;145
MLL	Mixed-lineage leukemia	11q23	Methyltransferase	2-3%	211
DNMT3A	DNA methyl transferase 3A	2p23	Methyltransferase	3-8%	96;97
NPM1	Nucleophosmin	5q35	Nucleocytoplasmic transport	<1-5%	138;233
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	11q23.3	E3 ubiquitin ligase, signal transduction	1-7%	138;234
IDH1	Isocitrate dehydrogenase 1	2q33.3	Metabolic pathways	2-4%	138;235-237
IDH2	Isocitrate dehydrogenase 2	15q26.1	Metabolic pathways	<1-5%	138;236;237
SF3B1 (and other spliceosome pathway genes)	Splicing factor 3B subunit 1	2q33.1	RNA splicing	20-30% (higher in subtypes with ringed sideroblasts)	238;239
TET2 **EZH2**	this thesis				

In addition to mutations, *TP53* can also be affected by other processes, such as a deletion of chromosome 17/17p, which occurs in approximately 5% of patients (**Figure 3**). Also, *TP53* can be indirectly involved in MDS pathogenesis as was shown for example in a mouse model of 5q-syndrome[108]. Similarly, the transcription factor *RUNX1* (*AML1*) can be affected in multiple ways. *RUNX1* is important in normal hematopoiesis and its function may be compromised in MDS by translocations involving its locus on chromosome 21 and by mutations. As applies to several other mutated genes in MDS, *RUNX1* mutations are detected more frequently in high risk MDS[138;139]. Mutations in several other transcription factors, CEBPA and *WT1,* are known to occur in MDS, but at a low frequency. On the contrary, overexpression of *WT1* is detectable in patients with MDS and correlates with a worse prognosis[140;141]. Mutations in *NPM1,* that occur in approximately 30% of AML patients, are detected in only a minority of MDS patients. However, since *NPM1* is located on chromosome 5q, haploinsufficiency of this gene may also play a role in the pathogenesis of MDS[142]. *NPM1* has been associated with a number of cellular processes, one of which is regulation of the p53 pathway[143]. Recently, mutations in several other genes have been identified in MDS. The mutational frequency of some of these (*BCL*, *IDH1* and *IDH2*) seems low, whereas mutations of the gene *ASXL1* were detected in 10-20% of MDS patients[138;144;145]. *ASXL1* is a member of the group of polycomb genes that are involved in regulation of transcription. *ASXL1* was found to be a member of a repressive complex containing histone H1.2[146]. However, it was also shown to have transcriptional activating properties[147]. In mice haploinsufficiency of *ASXL1* did not cause MDS, although hematopoiesis was affected[148]. This may be explained by the fact that the mutants used were not identical to the mutated genes found in MDS patients. Alternatively secondary defects are required to cause a clear phenotype.

In conclusion, a variety of cytogenetic defects has been found in hematopoietic cells of patients with MDS. Their discovery has improved understanding of MDS pathogenesis and has let in some instances to the application of specific pharmaceutical compounds in the treatment of MDS. However, in the majority of patients the genetic background of MDS remains unknown, mainly because only large or no genetic defects are detected.

Diagnosing and treating patients with MDS

Patients with MDS usually present with symptoms caused by anemia or, less often, another cytopenia. Morphological examination of the blood and bone marrow lays the foundation of the diagnosis of MDS, however, is often not sufficient for a definitive diagnosis. Dysplasia of at least 10% of the cells of the erythroid, monocytic, granulocytic or megakaryocytic lineage is the cardinal feature of MDS. Dysplasia includes features such as multinuclear erythroid precursors, hyposegmentation and hypogranulation of neutrophils and hypolobulation of megakaryocytes. The interpretation of dysplasia is complicated by the fact that it has many causes besides MDS. These include infections (e.g. parvovirus B19), drugs and toxic substances (e.g. chemotherapy, G-CSF), nutritional deficiencies (vitamine B12, folic acid) and congenital disorders (e.g. congenital dyserythropoiesis). In addition to

dysplasia, other morphological features are important, such as the number of blasts (very immature hematopoietic cells) in the blood and bone marrow and the presence of ringed sideroblasts. Apart from their role in diagnosing MDS, features like the presence of hypolobulated megakaryocytes and thrombocytosis may also indicate the possible presence of a specific MDS subtype, in this case 5q-syndrome. In addition, morphological characteristics are the main determinants of the classification of MDS in prognostically relevant groups. In some cases, particularly those with an increased number of blasts, the diagnosis of MDS can be made if the required 10% dysplasia is present and other causes of dysplasia have been excluded or are unlikely. In the other cases cytogenetic examination of the hematopoietic cells can provide further diagnostic information. As mentioned previously, karyotype analysis reveals chromosomal aberrations in approximately 50% of patients. Since the presence of these aberrations has prognostic implications as well, karyotype analysis is usually performed in all MDS patients. Occasionally, cytogenetic defects are found in patients with (a) cytopenia(s) without morphological abnormalities[149], requiring careful evaluation. Another way to confirm a suspected diagnosis of MDS is by histological examination of the bone marrow, which may exclude causes of non-MDS dysplasia and provide additional information on cellularity, clustering of blasts and fibrosis. In addition to the clinical characteristics, morphological and histological examination, other techniques such as flow cytometry and molecular genetic assays may facilitate a diagnosis of MDS. However, these techniques are not yet routinely used. By use of combinations of flow cytometric markers, dysplasia of the erythroid and myeloid cells can be evaluated [150;151]. In addition, aberrant expression of markers (increased or decreased expression of normal markers or expression of lineage-infidelity markers) has been shown to have prognostic implications[152;153]. Further standardization and validation of the flow cytometric parameters used to distinguish MDS from other hematopoietic disorders will facilitate the diagnostic process of MDS in the future. Recently, the results of the implementation of flow-cytometry in the diagnosis of MDS in the Netherlands have been published[154].

The routine application of molecular genetic techniques for example to detect mutated genes is limited by the fact that the mutations identified thus far are not specific for MDS and occur in only a minority of patients. Due to the limitations of the assays available for diagnosing MDS, a number of patients remains with a diagnosis of 'possible MDS' and requires accurate follow-up to evaluate the clinical course. Once the diagnosis of MDS has been established and an estimation of the prognosis has been made using the classification systems described before, several treatment options may be considered. Hematopoietic stem cell transplantation remains the only potentially curative treatment for MDS. Only a minority of patients are treated by stem cell transplantation, due to the toxicity of this treatment and the lack of available donors. Other treatment options include supportive therapy, such as erythrocyte transfusions and G-CSF and disease-modifying therapy. Disease-modifying therapies include for example ATG and lenalidomide. Most disease-modifying therapies are currently subject of extensive investigation to determine which patients are most likely to respond and to what extent combination therapy (for example a hypomethylating agent with an HDAC inhibitor) benefits patients.

Outline of this thesis

As discussed in the previous section, MDS is a heterogeneous group of diseases, complicating diagnosis, estimation of prognosis and development and proper application of novel treatment modalities. Improving insight into the pathogenesis of MDS and defining the biological pathways that are commonly disturbed in these patients could improve the quality of MDS patient care. The first part of this thesis focuses on deregulation of a biological pathway that is not thought to be the primary defect leading to MDS, but has often been related to development of the devastating cytopenias: apoptosis. This study aims at defining commonly deregulated apoptosis-related genes in isolated hematopoietic cells of MDS patients with various clinical phenotypes (**chapter 2**). The second part of this thesis describes the search for genetic defects that are potentially causally involved in the development of the malignant clone in MDS. The results of genome-wide analysis of genetic defects using single-nucleotide polymorphism (SNP) arrays (**Box 3**) in hematopoietic cells isolated from MDS patients is described in **chapter 3**. The identification of recurrent aberrations in the Ten-eleven-translocation-2 (*TET2*) gene in MDS that resulted from this analysis is described in chapter 3 as well. In **chapter 4**, the presence of *TET2* aberrations is studied in other hematopoietic malignancies than MDS, namely acute myeloid and lymphoid leukemia in children. In **chapter 5** we return to the SNP-array analysis described in Chapter 3 and explore another commonly affected region of the genome in MDS, leading to the discovery of recurrent aberrations in the Enhancer-of-Zeste-homolog-2 (*EZH2*) gene. Finally, **chapter 6** provides an overview and discussion of the implications of the research described in this thesis.

Box 3

Single nucleotide polymorphism (SNP) arrays allow the detection of deletions and amplifications in the DNA as well as areas showing loss-of-heterozygosity. SNP-array analysis starts with cleaving DNA isolated from cells into smaller fragments using a restriction enzyme (e.g. nspI, which cuts DNA at the recognition site 5'…RCATG*Y…3'/3'…Y*GTACR…5'). Subsequently, linkers are ligated to the DNA fragments, allowing primers to bind for the subsequent amplification by PCR. The amplified DNA fragments are purified, fragmented further by DNAse I and labeled. The labeled DNA is then hybridized to the SNP-array. The SNP-array contains oligonucleotide probes corresponding to DNA sequences containing SNPs. If the labeled DNA contains a specific SNP, the DNA will bind to the probe. If not, the DNA will be removed by several washing steps. By fluorescently labeling the bound DNA and scanning the array, the amount of DNA and the presence of specific SNPs can be determined. One of the advantages of SNP-array analysis and also array-comparative genomic hybridization (array-CGH) is the higher resolution compared to conventional karyotyping. Also, no dividing cells are necessary to perform the analysis. A disadvantage is that balanced chromosomal aberrations cannot be detected. Only SNP-array analysis allows the detection of copy-number neutral loss of heterozygosity due to mitotic recombination.

	karyotyping	array-CGH	SNP-array
Sample	cells in metaphase	DNA	DNA
Resolution	low	high (depending on no. of probes)	high (depending on no. of probes)
Detection of:			
small subclones	possibly	no	no
copy number abnormalities	yes	yes	yes
balanced chromosomal aberrations	yes	no	no
uniparental disomy	no	no	yes

Reference List

1. Abramson S, Miller RG, Phillips RA. The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J.Exp.Med. 1977;145:1567-1579.
2. Keller G, Paige C, Gilboa E, Wagner EF. Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. Nature 1985;318:149-154.
3. Smith LG, Weissman IL, Heimfeld S. Clonal analysis of hematopoietic stem-cell differentiation in vivo. Proc.Natl.Acad.Sci.U.S.A 1991;88:2788-2792.
4. Dick JE, Magli MC, Huszar D, Phillips RA, Bernstein A. Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. Cell 1985;42:71-79.
5. Bell JJ, Bhandoola A. The earliest thymic progenitors for T cells possess myeloid lineage potential. Nature 2008;452:764-767.
6. Wada H, Masuda K, Satoh R et al. Adult T-cell progenitors retain myeloid potential. Nature 2008;452:768-772.
7. Adolfsson J, Mansson R, Buza-Vidas N et al. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 2005;121:295-306.
8. Benz C, Bleul CC. A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision. J.Exp.Med. 2005;202:21-31.
9. Lu M, Kawamoto H, Katsube Y, Ikawa T, Katsura Y. The common myelolymphoid progenitor: a key intermediate stage in hemopoiesis generating T and B cells. J.Immunol. 2002;169:3519-3525.
10. Doulatov S, Notta F, Eppert K et al. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nat.Immunol. 2010;11:585-593.
11. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. Nat.Rev.Immunol. 2006;6:93-106.
12. Zhang J, Niu C, Ye L et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003;425:836-841.
13. Xie Y, Yin T, Wiegraebe W et al. Detection of functional haematopoietic stem cell niche using real-time imaging. Nature 2009;457:97-101.
14. Lo CC, Fleming HE, Wu JW et al. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature 2009;457:92-96.
15. Rizo A, Vellenga E, de HG, Schuringa JJ. Signaling pathways in self-renewing hematopoietic and leukemic stem cells: do all stem cells need a niche? Hum.Mol.Genet. 2006;15 Spec No 2:R210-R219.
16. Reya T, Duncan AW, Ailles L et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003;423:409-414.
17. Kaushansky K. Lineage-specific hematopoietic growth factors. N.Engl.J.Med. 2006;354:2034-2045.
18. Nutt SL, Heavey B, Rolink AG, Busslinger M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature 1999;401:556-562.
19. Cobaleda C, Schebesta A, Delogu A, Busslinger M. Pax5: the guardian of B cell identity and function. Nat. Immunol. 2007;8:463-470.

20. McKercher SR, Torbett BE, Anderson KL et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J. 1996;15:5647-5658.

21. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 1994;265:1573-1577.

22. Hohaus S, Petrovick MS, Voso MT et al. PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. Mol.Cell Biol. 1995;15:5830-5845.

23. Kamath MB, Houston IB, Janovski AJ et al. Dose-dependent repression of T-cell and natural killer cell genes by PU.1 enforces myeloid and B-cell identity. Leukemia 2008;22:1214-1225.

24. Friedman AD. Transcriptional control of granulocyte and monocyte development. Oncogene 2007;26:6816-6828.

25. Chung YS, Kim HJ, Kim TM et al. Undifferentiated hematopoietic cells are characterized by a genome-wide undermethylation dip around the transcription start site and a hierarchical epigenetic plasticity. Blood 2009;114:4968-4978.

26. Cui K, Zang C, Roh TY et al. Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. Cell Stem Cell 2009;4:80-93.

27. Weishaupt H, Sigvardsson M, Attema JL. Epigenetic chromatin states uniquely define the developmental plasticity of murine hematopoietic stem cells. Blood 2010;115:247-256.

28. Maes J, Maleszewska M, Guillemin C et al. Lymphoid-affiliated genes are associated with active histone modifications in human hematopoietic stem cells. Blood 2008;112:2722-2729.

29. Vardiman JW, Thiele J, Arber DA et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009;114:937-951.

30. Bennett JM, Catovsky D, Daniel MT et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br.J.Haematol. 1976;33:451-458.

31. Bennett JM, Catovsky D, Daniel MT et al. Proposals for the classification of the myelodysplastic syndromes. Br.J.Haematol. 1982;51:189-199.

32. Kerkhofs H, Hermans J, Haak HL, Leeksma CH. Utility of the FAB classification for myelodysplastic syndromes: investigation of prognostic factors in 237 cases. Br.J.Haematol. 1987;65:73-81.

33. Michaux JL, Martiat P. Chronic myelomonocytic leukaemia (CMML)--a myelodysplastic or myeloproliferative syndrome? Leuk.Lymphoma 1993;9:35-41.

34. Germing U, Gattermann N, Minning H, Heyll A, Aul C. Problems in the classification of CMML--dysplastic versus proliferative type. Leuk.Res. 1998;22:871-878.

35. Seymour JF, Estey EH. The contribution of Auer rods to the classification and prognosis of myelodysplastic syndromes. Leuk.Lymphoma 1995;17:79-85.

36. Greenberg P, Cox C, LeBeau MM et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997;89:2079-2088.

37. Germing U, Gattermann N, Strupp C, Aivado M, Aul C. Validation of the WHO proposals for a new classification of primary myelodysplastic syndromes: a retrospective analysis of 1600 patients. Leuk.Res. 2000;24:983-992.

38. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 2002;100:2292-2302.

39. Howe RB, Porwit-MacDonald A, Wanat R, Tehranchi R, Hellstrom-Lindberg E. The WHO classification of MDS does make a difference. Blood 2004;103:3265-3270.

40. Verburgh E, Achten R, Louw VJ et al. A new disease categorization of low-grade myelodysplastic syndromes based on the expression of cytopenia and dysplasia in one versus more than one lineage improves on the WHO classification. Leukemia 2007;21:668-677.

41. Malcovati L, Germing U, Kuendgen A et al. Time-dependent prognostic scoring system for predicting survival and leukemic evolution in myelodysplastic syndromes. J.Clin.Oncol. 2007;25:3503-3510.

42. Kantarjian H, O'Brien S, Ravandi F et al. Proposal for a new risk model in myelodysplastic syndrome that accounts for events not considered in the original International Prognostic Scoring System. Cancer 2008;113:1351-1361.

43. Garcia-Manero G, Shan J, Faderl S et al. A prognostic score for patients with lower risk myelodysplastic syndrome. Leukemia 2008;22:538-543.

44. la Porta MG, Malcovati L, Boveri E et al. Clinical relevance of bone marrow fibrosis and CD34-positive cell clusters in primary myelodysplastic syndromes. J.Clin.Oncol. 2009;27:754-762.

45. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. N.Engl.J.Med. 2009;361:1570-1583.

46. Houwerzijl EJ, Blom NR, van der Want JJ, Vellenga E, de Wolf JT. Megakaryocytic dysfunction in myelodysplastic syndromes and idiopathic thrombocytopenic purpura is in part due to different forms of cell death. Leukemia 2006;20:1937-1942.

47. Raza A, Gezer S, Mundle S et al. Apoptosis in bone marrow biopsy samples involving stromal and hematopoietic cells in 50 patients with myelodysplastic syndromes. Blood 1995;86:268-276.

48. Raza A, Mundle S, Iftikhar A et al. Simultaneous assessment of cell kinetics and programmed cell death in bone marrow biopsies of myelodysplastics reveals extensive apoptosis as the probable basis for ineffective hematopoiesis. Am.J.Hematol. 1995;48:143-154.

49. Tsoplou P, Kouraklis-Symeonidis A, Thanopoulou E et al. Apoptosis in patients with myelodysplastic syndromes: differential involvement of marrow cells in 'good' versus 'poor' prognosis patients and correlation with apoptosis-related genes. Leukemia 1999;13:1554-1563.

50. Bogdanovic AD, Trpinac DP, Jankovic GM et al. Incidence and role of apoptosis in myelodysplastic syndrome: morphological and ultrastructural assessment. Leukemia 1997;11:656-659.

51. Hellstrom-Lindberg E, Kanter-Lewensohn L, Ost A. Morphological changes and apoptosis in bone marrow from patients with myelodysplastic syndromes treated with granulocyte-CSF and erythropoietin. Leuk.Res. 1997;21:415-425.

52. Parker JE, Mufti GJ, Rasool F et al. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. Blood 2000;96:3932-3938.

53. Lin CW, Manshouri T, Jilani I et al. Proliferation and apoptosis in acute and chronic leukemias and myelodysplastic syndrome. Leuk.Res. 2002;26:551-559.

54. Huh YO, Jilani I, Estey E et al. More cell death in refractory anemia with excess blasts in transformation than in acute myeloid leukemia. Leukemia 2002;16:2249-2252.

55. Suarez L, Vidriales MB, Garcia-Larana J et al. CD34+ cells from acute myeloid leukemia, myelodysplastic syndromes, and normal bone marrow display different apoptosis and drug resistance-associated phenotypes. Clin.Cancer Res. 2004;10:7599-7606.

56. Suarez L, Vidriales MB, Sanz G et al. Expression of APO2.7, bcl-2 and bax apoptosis-associated proteins in CD34- bone marrow cell compartments from patients with myelodysplastic syndromes. Leukemia 2004;18:1311-1313.

57. Rajapaksa R, Ginzton N, Rott LS, Greenberg PL. Altered oncoprotein expression and apoptosis in myelodysplastic syndrome marrow cells. Blood 1996;88:4275-4287.

58. Boudard D, Vasselon C, Bertheas MF et al. Expression and prognostic significance of Bcl-2 family proteins in myelodysplastic syndromes. Am.J.Hematol. 2002;70:115-125.

59. Maiuri MC, Tasdemir E, Criollo A et al. Control of autophagy by oncogenes and tumor suppressor genes. Cell Death.Differ. 2009;16:87-93.

60. Houwerzijl EJ, Pol HW, Blom NR et al. Erythroid precursors from patients with low-risk myelodysplasia demonstrate ultrastructural features of enhanced autophagy of mitochondria. Leukemia 2009;23:886-891.

61. Kroef MJ, Bolk MJ, Muus P et al. Mosaicism of the 5q deletion as assessed by interphase FISH is a common phenomenon in MDS and restricted to myeloid cells. Leukemia 1997;11:519-523.

62. van KH, Fibbe WE, Jansen RP et al. Clonal involvement of granulocytes and monocytes, but not of T and B lymphocytes and natural killer cells in patients with myelodysplasia: analysis by X-linked restriction fragment length polymorphisms and polymerase chain reaction of the phosphoglycerate kinase gene. Blood 1992;80:1774-1780.

63. Meers S, Vandenberghe P, Boogaerts M, Verhoef G, Delforge M. The clinical significance of activated lymphocytes in patients with myelodysplastic syndromes: a single centre study of 131 patients. Leuk.Res. 2008;32:1026-1035.

64. Gerritsen WR, Donohue J, Bauman J et al. Clonal analysis of myelodysplastic syndrome: monosomy 7 is expressed in the myeloid lineage, but not in the lymphoid lineage as detected by fluorescent in situ hybridization. Blood 1992;80:217-224.

65. Kitagawa M, Yamaguchi S, Takahashi M et al. Localization of Fas and Fas ligand in bone marrow cells demonstrating myelodysplasia. Leukemia 1998;12:486-492.

66. Bouscary D, De VJ, Guesnu M et al. Fas/Apo-1 (CD95) expression and apoptosis in patients with myelodysplastic syndromes. Leukemia 1997;11:839-845.

67. Gersuk GM, Beckham C, Loken MR et al. A role for tumour necrosis factor-alpha, Fas and Fas-Ligand in marrow failure associated with myelodysplastic syndrome. Br.J.Haematol. 1998;103:176-188.

68. Claessens YE, Bouscary D, Dupont JM et al. In vitro proliferation and differentiation of erythroid progenitors from patients with myelodysplastic syndromes: evidence for Fas-dependent apoptosis. Blood 2002;99:1594-1601.

69. Epperson DE, Nakamura R, Sauntharajah Y, Melenhorst J, Barrett AJ. Oligoclonal T cell expansion in myelodysplastic syndrome: evidence for an autoimmune process. Leuk.Res. 2001;25:1075-1083.

70. Melenhorst JJ, Eniafe R, Follmann D et al. Molecular and flow cytometric characterization of the CD4 and CD8 T-cell repertoire in patients with myelodysplastic syndrome. Br.J.Haematol. 2002;119:97-105.

71. Epling-Burnette PK, Painter JS, Rollison DE et al. Prevalence and clinical association of clonal T-cell expansions in Myelodysplastic Syndrome. Leukemia 2007;21:659-667.

72. Fozza C, Contini S, Galleu A et al. Patients with myelodysplastic syndromes display several T-cell expansions, which are mostly polyclonal in the CD4(+) subset and oligoclonal in the CD8(+) subset. Exp.Hematol. 2009;37:947-955.

73. Kook H, Zeng W, Guibin C et al. Increased cytotoxic T cells with effector phenotype in aplastic anemia and myelodysplasia. Exp.Hematol. 2001;29:1270-1277.

74. Kordasti SY, Afzali B, Lim Z et al. IL-17-producing CD4(+) T cells, pro-inflammatory cytokines and apoptosis are increased in low risk myelodysplastic syndrome. Br.J.Haematol. 2009;145:64-72.

75. Molldrem JJ, Leifer E, Bahceci E et al. Antithymocyte globulin for treatment of the bone marrow failure associated with myelodysplastic syndromes. Ann.Intern.Med. 2002;137:156-163.

76. Steensma DP, Dispenzieri A, Moore SB, Schroeder G, Tefferi A. Antithymocyte globulin has limited efficacy and substantial toxicity in unselected anemic patients with myelodysplastic syndrome. Blood 2003;101:2156-2158.

77. Killick SB, Mufti G, Cavenagh JD et al. A pilot study of antithymocyte globulin (ATG) in the treatment of patients with 'low-risk' myelodysplasia. Br.J.Haematol. 2003;120:679-684.

78. Yazji S, Giles FJ, Tsimberidou AM et al. Antithymocyte globulin (ATG)-based therapy in patients with myelodysplastic syndromes. Leukemia 2003;17:2101-2106.

79. Selleri C, Maciejewski JP, Catalano L et al. Effects of cyclosporine on hematopoietic and immune functions in patients with hypoplastic myelodysplasia: in vitro and in vivo studies. Cancer 2002;95:1911-1922.

80. Molldrem JJ, Jiang YZ, Stetler-Stevenson M et al. Haematological response of patients with myelodysplastic syndrome to antithymocyte globulin is associated with a loss of lymphocyte-mediated inhibition of CFU-GM and alterations in T-cell receptor Vbeta profiles. Br.J.Haematol. 1998;102:1314-1322.

81. Kochenderfer JN, Kobayashi S, Wieder ED, Su C, Molldrem JJ. Loss of T-lymphocyte clonal dominance in patients with myelodysplastic syndrome responsive to immunosuppression. Blood 2002;100:3639-3645.

82. Kordasti SY, Ingram W, Hayden J et al. CD4+CD25high Foxp3+ regulatory T cells in myelodysplastic syndrome (MDS). Blood 2007;110:847-850.

83. Alfinito F, Sica M, Luciano L et al. Immune dysregulation and dyserythropoiesis in the myelodysplastic syndromes. Br.J.Haematol. 2010;148:90-98.

84. Kondo A, Yamashita T, Tamura H et al. Interferon-gamma and tumor necrosis factor-alpha induce an immunoinhibitory molecule, B7-H1, via nuclear factor-kappaB activation in blasts in myelodysplastic syndromes. Blood 2010;116:1124-1131.

85. Marcondes AM, Mhyre AJ, Stirewalt DL et al. Dysregulation of IL-32 in myelodysplastic syndrome and chronic myelomonocytic leukemia modulates apoptosis and impairs NK function. Proc.Natl.Acad.Sci.U.S.A 2008;105:2865-2870.

86. Epling-Burnette PK, Bai F, Painter JS et al. Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors. Blood 2007;109:4816-4824.

87. Grovdal M, Khan R, Aggerholm A et al. Negative effect of DNA hypermethylation on the outcome of intensive chemotherapy in older patients with high-risk myelodysplastic syndromes and acute myeloid leukemia following myelodysplastic syndrome. Clin.Cancer Res. 2007;13:7107-7112.

88. Shen L, Kantarjian H, Guo Y et al. DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. J.Clin.Oncol. 2010;28:605-613.

89. Uchida T, Kinoshita T, Nagai H et al. Hypermethylation of the p15INK4B gene in myelodysplastic syndromes. Blood 1997;90:1403-1409.

90. Quesnel B, Guillerm G, Vereecque R et al. Methylation of the p15(INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression. Blood 1998;91:2985-2990.

91. Romermann D, Hasemeier B, Metzig K et al. Global increase in DNA methylation in patients with myelodysplastic syndrome. Leukemia 2008;22:1954-1956.

92. Jiang Y, Dunbar A, Gondek LP et al. Aberrant DNA methylation is a dominant mechanism in MDS progression to AML. Blood 2009;113:1315-1325.

93. Figueroa ME, Skrabanek L, Li Y et al. MDS and secondary AML display unique patterns and abundance of aberrant DNA methylation. Blood 2009

94. Langer F, Dingemann J, Kreipe H, Lehmann U. Up-regulation of DNA methyltransferases DNMT1, 3A, and 3B in myelodysplastic syndrome. Leuk.Res. 2005;29:325-329.

95. Hopfer O, Komor M, Koehler IS et al. Aberrant promotor methylation in MDS hematopoietic cells during in vitro lineage specific differentiation is differently associated with DNMT isoforms. Leuk.Res. 2009;33:434-442.

96. Thol F, Winschel C, Ludeking A et al. Rare occurence of DNMT3A mutations in myelodysplastic syndromes. Haematologica 2011

97. Walter MJ, Ding L, Shen D et al. Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. Leukemia 2011;25:1153-1158.

98. Spensberger D, Delwel R. A novel interaction between the proto-oncogene Evi1 and histone methyltransferases, SUV39H1 and G9a. FEBS Lett. 2008;582:2761-2767.

99. Cattaneo F, Nucifora G. EVI1 recruits the histone methyltransferase SUV39H1 for transcription repression. J.Cell Biochem. 2008;105:344-352.

100. Griffiths EA, Gore SD. DNA methyltransferase and histone deacetylase inhibitors in the treatment of myelodysplastic syndromes. Semin.Hematol. 2008;45:23-30.

101. Haase D, Germing U, Schanz J et al. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. Blood 2007;110:4385-4395.

102. Boultwood J, Fidler C, Strickson AJ et al. Narrowing and genomic annotation of the commonly deleted region of the 5q- syndrome. Blood 2002;99:4638-4641.

103. Le Beau MM, Espinosa R, III, Neuman WL et al. Cytogenetic and molecular delineation of the smallest commonly deleted region of chromosome 5 in malignant myeloid diseases. Proc.Natl.Acad.Sci.U.S.A 1993;90:5484-5488.

104. Zhao N, Stoffel A, Wang PW et al. Molecular delineation of the smallest commonly deleted region of chromosome 5 in malignant myeloid diseases to 1-1.5 Mb and preparation of a PAC-based physical map. Proc. Natl.Acad.Sci.U.S.A 1997;94:6948-6953.

105. Horrigan SK, Arbieva ZH, Xie HY et al. Delineation of a minimal interval and identification of 9 candidates for a tumor suppressor gene in malignant myeloid disorders on 5q31. Blood 2000;95:2372-2377.

106. Lai F, Godley LA, Joslin J et al. Transcript map and comparative analysis of the 1.5-Mb commonly deleted segment of human 5q31 in malignant myeloid diseases with a del(5q). Genomics 2001;71:235-245.

107. Ebert BL, Pretz J, Bosco J et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. Nature 2008;451:335-339.

108. Barlow JL, Drynan LF, Hewett DR et al. A p53-dependent mechanism underlies macrocytic anemia in a mouse model of human 5q- syndrome. Nat.Med. 2010;16:59-66.

109. Starczynowski DT, Kuchenbauer F, Argiropoulos B et al. Identification of miR-145 and miR-146a as mediators of the 5q- syndrome phenotype. Nat.Med. 2010;16:49-58.

110. Liu TX, Becker MW, Jelinek J et al. Chromosome 5q deletion and epigenetic suppression of the gene encoding alpha-catenin (CTNNA1) in myeloid cell transformation. Nat.Med. 2007;13:78-83.

111. Xie H, Hu Z, Chyna B, Horrigan SK, Westbrook CA. Human mortalin (HSPA9): a candidate for the myeloid leukemia tumor suppressor gene on 5q31. Leukemia 2000;14:2128-2134.

112. Craven SE, French D, Ye W, de SF, Rosenthal A. Loss of Hspa9b in zebrafish recapitulates the ineffective hematopoiesis of the myelodysplastic syndrome. Blood 2005;105:3528-3534.

113. Joslin JM, Fernald AA, Tennant TR et al. Haploinsufficiency of EGR1, a candidate gene in the del(5q), leads to the development of myeloid disorders. Blood 2007;110:719-726.

114. Le Beau MM, Espinosa R, III, Davis EM et al. Cytogenetic and molecular delineation of a region of chromosome 7 commonly deleted in malignant myeloid diseases. Blood 1996;88:1930-1935.

115. Johnson EJ, Scherer SW, Osborne L et al. Molecular definition of a narrow interval at 7q22.1 associated with myelodysplasia. Blood 1996;87:3579-3586.

116. Fischer K, Frohling S, Scherer SW et al. Molecular cytogenetic delineation of deletions and translocations involving chromosome band 7q22 in myeloid leukemias. Blood 1997;89:2036-2041.

117. Liang H, Fairman J, Claxton DF et al. Molecular anatomy of chromosome 7q deletions in myeloid neoplasms: evidence for multiple critical loci. Proc.Natl.Acad.Sci.U.S.A 1998;95:3781-3785.

118. Dohner K, Brown J, Hehmann U et al. Molecular cytogenetic characterization of a critical region in bands 7q35-q36 commonly deleted in malignant myeloid disorders. Blood 1998;92:4031-4035.

119. Tosi S, Scherer SW, Giudici G et al. Delineation of multiple deleted regions in 7q in myeloid disorders. Genes Chromosomes.Cancer 1999;25:384-392.

120. Curtiss NP, Bonifas JM, Lauchle JO et al. Isolation and analysis of candidate myeloid tumor suppressor genes from a commonly deleted segment of 7q22. Genomics 2005;85:600-607.

121. Roulston D, Espinosa R, III, Stoffel M, Bell GI, Le Beau MM. Molecular genetics of myeloid leukemia: identification of the commonly deleted segment of chromosome 20. Blood 1993;82:3424-3429.

122. Wang PW, Eisenbart JD, Espinosa R, III et al. Refinement of the smallest commonly deleted segment of chromosome 20 in malignant myeloid diseases and development of a PAC-based physical and transcription map. Genomics 2000;67:28-39.

123. Wang PW, Iannantuoni K, Davis EM et al. Refinement of the commonly deleted segment in myeloid leukemias with a del(20q). Genes Chromosomes.Cancer 1998;21:75-81.

124. Bench AJ, Aldred MA, Humphray SJ et al. A detailed physical and transcriptional map of the region of chromosome 20 that is deleted in myeloproliferative disorders and refinement of the common deleted region. Genomics 1998;49:351-362.

125. Douet-Guilbert N, Basinko A, Morel F et al. Chromosome 20 deletions in myelodysplastic syndromes and Philadelphia-chromosome-negative myeloproliferative disorders: characterization by molecular cytogenetics of commonly deleted and retained regions. Ann.Hematol. 2008;87:537-544.

126. Huh J, Tiu RV, Gondek LP et al. Characterization of chromosome arm 20q abnormalities in myeloid malignancies using genome-wide single nucleotide polymorphism array analysis. Genes Chromosomes.Cancer 2010;49:390-399.

127. List A, Dewald G, Bennett J et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. N.Engl.J.Med. 2006;355:1456-1465.

128. Steensma DP, Neiger JD, Porcher JC et al. Rearrangements and amplification of IER3 (IEX-1) represent a novel and recurrent molecular abnormality in myelodysplastic syndromes. Cancer Res. 2009;69:7518-7523.

129. Hirai H, Kobayashi Y, Mano H et al. A point mutation at codon 13 of the N-ras oncogene in myelodysplastic syndrome. Nature 1987;327:430-432.

130. Liu E, Hjelle B, Morgan R, Hecht F, Bishop JM. Mutations of the Kirsten-ras proto-oncogene in human preleukaemia. Nature 1987;330:186-188.

131. Padua RA, Guinn BA, Al-Sabah AI et al. RAS, FMS and p53 mutations and poor clinical outcome in myelodysplasias: a 10-year follow-up. Leukemia 1998;12:887-892.

132. Shih LY, Huang CF, Wang PN et al. Acquisition of FLT3 or N-ras mutations is frequently associated with progression of myelodysplastic syndrome to acute myeloid leukemia. Leukemia 2004;18:466-475.

133. Pinheiro RF, de Sa ME, Silva MR, Alberto FL, Chauffaille ML. FLT3 internal tandem duplication during myelodysplastic syndrome follow-up: a marker of transformation to acute myeloid leukemia. Cancer Genet. Cytogenet. 2008;183:89-93.

134. Braun T, Fenaux P. Farnesyltransferase inhibitors and their potential role in therapy for myelodysplastic syndromes and acute myeloid leukaemia. Br.J.Haematol. 2008;141:576-586.

135. Szpurka H, Tiu R, Murugesan G et al. Refractory anemia with ringed sideroblasts associated with marked thrombocytosis (RARS-T), another myeloproliferative condition characterized by JAK2 V617F mutation. Blood 2006;108:2173-2181.

136. Wang SA, Hasserjian RP, Loew JM et al. Refractory anemia with ringed sideroblasts associated with marked thrombocytosis harbors JAK2 mutation and shows overlapping myeloproliferative and myelodysplastic features. Leukemia 2006;20:1641-1644.

137. Kita-Sasai Y, Horiike S, Misawa S et al. International prognostic scoring system and TP53 mutations are independent prognostic indicators for patients with myelodysplastic syndrome. Br.J.Haematol. 2001;115:309-312.

138. Rocquain J, Carbuccia N, Trouplin V et al. Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. BMC.Cancer 2010;10:401.

139. Harada H, Harada Y, Niimi H et al. High incidence of somatic mutations in the AML1/RUNX1 gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia. Blood 2004;103:2316-2324.

140. Tamaki H, Ogawa H, Ohyashiki K et al. The Wilms' tumor gene WT1 is a good marker for diagnosis of disease progression of myelodysplastic syndromes. Leukemia 1999;13:393-399.

141. Patmasiriwat P, Fraizer G, Kantarjian H, Saunders GF. WT1 and GATA1 expression in myelodysplastic syndrome and acute leukemia. Leukemia 1999;13:891-900.

142. Sportoletti P, Grisendi S, Majid SM et al. Npm1 is a haploinsufficient suppressor of myeloid and lymphoid malignancies in the mouse. Blood 2008;111:3859-3862.

143. Colombo E, Marine JC, Danovi D, Falini B, Pelicci PG. Nucleophosmin regulates the stability and transcriptional activity of p53. Nat.Cell Biol. 2002;4:529-533.

144. Gelsi-Boyer V, Trouplin V, Adelaide J et al. Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. Br.J.Haematol. 2009;145:788-800.

145. Boultwood J, Perry J, Pellagatti A et al. Frequent mutation of the polycomb-associated gene ASXL1 in the myelodysplastic syndromes and in acute myeloid leukemia. Leukemia 2010;24:1062-1065.

146. Kim K, Choi J, Heo K et al. Isolation and characterization of a novel H1.2 complex that acts as a repressor of p53-mediated transcription. J.Biol.Chem. 2008;283:9113-9126.

147. Cho YS, Kim EJ, Park UH, Sin HS, Um SJ. Additional sex comb-like 1 (ASXL1), in cooperation with SRC-1, acts as a ligand-dependent coactivator for retinoic acid receptor. J.Biol.Chem. 2006;281:17588-17598.

148. Fisher CL, Pineault N, Brookes C et al. Loss-of-function Additional sex combs like 1 mutations disrupt hematopoiesis but do not cause severe myelodysplasia or leukemia. Blood 2010;115:38-46.

149. Steensma DP, Dewald GW, Hodnefield JM, Tefferi A, Hanson CA. Clonal cytogenetic abnormalities in bone marrow specimens without clear morphologic evidence of dysplasia: a form fruste of myelodysplasia? Leuk. Res. 2003;27:235-242.

150. Malcovati L, la Porta MG, Lunghi M et al. Flow cytometry evaluation of erythroid and myeloid dysplasia in patients with myelodysplastic syndrome. Leukemia 2005;19:776-783.

151. Matarraz S, Lopez A, Barrena S et al. The immunophenotype of different immature, myeloid and B-cell lineage-committed CD34+ hematopoietic cells allows discrimination between normal/reactive and myelodysplastic syndrome precursors. Leukemia 2008;22:1175-1183.

152. Ogata K, Nakamura K, Yokose N et al. Clinical significance of phenotypic features of blasts in patients with myelodysplastic syndrome. Blood 2002;100:3887-3896.

153. van de Loosdrecht AA, Westers TM, Westra AH et al. Identification of distinct prognostic subgroups in low- and intermediate-1-risk myelodysplastic syndromes by flow cytometry. Blood 2008;111:1067-1077.

154. Westers TM, van dV, V, Alhan C et al. Implementation of flow cytometry in the diagnostic work-up of myelodysplastic syndromes in a multicenter approach: Report from the Dutch Working Party on Flow Cytometry in MDS. Leuk.Res. 2011

155. Dhodapkar M, Grill J, Lust JA. Abnormal regional hypermethylation of the calcitonin gene in myelodysplastic syndromes. Leuk.Res. 1995;19:719-726.

156. Ihalainen J, Pakkala S, Savolainen ER, Jansson SE, Palotie A. Hypermethylation of the calcitonin gene in the myelodysplastic syndromes. Leukemia 1993;7:263-267.

157. Aggerholm A, Holm MS, Guldberg P, Olesen LH, Hokland P. Promoter hypermethylation of p15INK4B, HIC1, CDH1, and ER is frequent in myelodysplastic syndrome and predicts poor prognosis in early-stage patients. Eur.J.Haematol. 2006;76:23-32.

158. Greco M, D'Alo F, Scardocci A et al. Promoter methylation of DAPK1, E-cadherin and thrombospondin-1 in de novo and therapy-related myeloid neoplasms. Blood Cells Mol.Dis. 2010;45:181-185.

159. Fandy TE, Herman JG, Kerns P et al. Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies. Blood 2009;114:2764-2773.

160. Aoki E, Uchida T, Ohashi H et al. Methylation status of the p15INK4B gene in hematopoietic progenitors and peripheral blood cells in myelodysplastic syndromes. Leukemia 2000;14:586-593.

161. Tien HF, Tang JH, Tsay W et al. Methylation of the p15(INK4B) gene in myelodysplastic syndrome: it can be detected early at diagnosis or during disease progression and is highly associated with leukaemic transformation. Br.J.Haematol. 2001;112:148-154.

162. Teofili L, Martini M, Di MA et al. Expression of p15(ink4b) gene during megakaryocytic differentiation of normal and myelodysplastic hematopoietic progenitors. Blood 2001;98:495-497.

163. Nguyen C, Liang G, Nguyen TT et al. Susceptibility of nonpromoter CpG islands to de novo methylation in normal and neoplastic cells. J.Natl.Cancer Inst. 2001;93:1465-1472.

164. Preisler HD, Li B, Chen H et al. P15INK4B gene methylation and expression in normal, myelodysplastic, and acute myelogenous leukemia cells and in the marrow cells of cured lymphoma patients. Leukemia 2001;15:1589-1595.

165. Daskalakis M, Nguyen TT, Nguyen C et al. Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. Blood 2002;100:2957-2964.

166. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Methylation of p15INK4B is common, is associated with deletion of genes on chromosome arm 7q and predicts a poor prognosis in therapy-related myelodysplasia and acute myeloid leukemia. Leukemia 2003;17:1813-1819.

167. Hofmann WK, Takeuchi S, Takeuchi N et al. Comparative analysis of hypermethylation of cell cycle control and DNA-mismatch repair genes in low-density and CD34+ bone marrow cells from patients with myelodysplastic syndrome. Leuk.Res. 2006;30:1347-1353.

168. Kim M, Oh B, Kim SY et al. p15INK4b methylation correlates with thrombocytopenia, blast percentage, and survival in myelodysplastic syndromes in a dose dependent manner: quantitation using pyrosequencing study. Leuk.Res. 2010;34:718-722.

169. Ye Y, McDevitt MA, Guo M et al. Progressive chromatin repression and promoter methylation of CTNNA1 associated with advanced myeloid malignancies. Cancer Res. 2009;69:8482-8490.

170. Voso MT, Scardocci A, Guidi F et al. Aberrant methylation of DAP-kinase in therapy-related acute myeloid leukemia and myelodysplastic syndromes. Blood 2004;103:698-700.

171. Qian J, Yao DM, Lin J et al. Methylation of DAPK1 promoter: frequent but not an adverse prognostic factor in myelodysplastic syndrome. Int.J.Lab Hematol. 2010;32:74-81.

172. Lin J, Wang YL, Qian J et al. Aberrant methylation of DNA-damage-inducible transcript 3 promoter is a common event in patients with myelodysplastic syndrome. Leuk.Res. 2010;34:991-994.

173. Iwai M, Kiyoi H, Ozeki K et al. Expression and methylation status of the FHIT gene in acute myeloid leukemia and myelodysplastic syndrome. Leukemia 2005;19:1367-1375.

174. Lin J, Yao DM, Qian J et al. Methylation status of fragile histidine triad (FHIT) gene and its clinical impact on prognosis of patients with myelodysplastic syndrome. Leuk.Res. 2008;32:1541-1545.

175. Bojesen SE, Ammerpohl O, Weinhausl A et al. Characterisation of the GRAF gene promoter and its methylation in patients with acute myeloid leukaemia and myelodysplastic syndrome. Br.J.Cancer 2006;94:323-332.

176. Qian J, Qian Z, Lin J et al. Aberrant methylation of GTPase regulator associated with the focal adhesion kinase (GRAF) promoter is an adverse prognostic factor in myelodysplastic syndrome. Eur.J.Haematol. 2010;85:174-176.

177. Wang H, Wang XQ, Xu XP, Lin GW. ID4 methylation predicts high risk of leukemic transformation in patients with myelodysplastic syndrome. Leuk.Res. 2010;34:598-604.

178. Potapova A, Hasemeier B, Romermann D et al. Epigenetic inactivation of tumour suppressor gene KLF11 in myelodysplastic syndromes*. Eur.J.Haematol. 2010;84:298-303.

179. Benetatos L, Hatzimichael E, Dasoula A et al. CpG methylation analysis of the MEG3 and SNRPN imprinted genes in acute myeloid leukemia and myelodysplastic syndromes. Leuk.Res. 2010;34:148-153.

180. Johan MF, Bowen DT, Frew ME, Goodeve AC, Reilly JT. Aberrant methylation of the negative regulators RASSFIA, SHP-1 and SOCS-1 in myelodysplastic syndromes and acute myeloid leukaemia. Br.J.Haematol. 2005;129:60-65.

181. Boumber YA, Kondo Y, Chen X et al. RIL, a LIM gene on 5q31, is silenced by methylation in cancer and sensitizes cancer cells to apoptosis. Cancer Res. 2007;67:1997-2005.

182. Brakensiek K, Langer F, Schlegelberger B, Kreipe H, Lehmann U. Hypermethylation of the suppressor of cytokine signalling-1 (SOCS-1) in myelodysplastic syndrome. Br.J.Haematol. 2005;130:209-217.

183. Wu SJ, Yao M, Chou WC et al. Clinical implications of SOCS1 methylation in myelodysplastic syndrome. Br.J.Haematol. 2006;135:317-323.

184. Haase D. Cytogenetic features in myelodysplastic syndromes. Ann.Hematol. 2008;87:515-526.

185. Janssen H, Wlodarska I, Mecucci C et al. Fusion of ETV6 to GOT1 in a case with myelodysplastic syndrome and t(10;12)(q24;p13). Haematologica 2006;91:949-951.

186. Raynaud SD, Baens M, Grosgeorge J et al. Fluorescence in situ hybridization analysis of t(3; 12)(q26; p13): a recurring chromosomal abnormality involving the TEL gene (ETV6) in myelodysplastic syndromes. Blood 1996;88:682-689.

187. Poppe B, Dastugue N, Vandesompele J et al. EVI1 is consistently expressed as principal transcript in common and rare recurrent 3q26 rearrangements. Genes Chromosomes.Cancer 2006;45:349-356.

188. Gamou T, Kitamura E, Hosoda F et al. The partner gene of AML1 in t(16;21) myeloid malignancies is a novel member of the MTG8(ETO) family. Blood 1998;91:4028-4037.

189. Nucifora G, Begy CR, Kobayashi H et al. Consistent intergenic splicing and production of multiple transcripts between AML1 at 21q22 and unrelated genes at 3q26 in (3;21)(q26;q22) translocations. Proc.Natl. Acad.Sci.U.S.A 1994;91:4004-4008.

190. Taki T, Sako M, Tsuchida M, Hayashi Y. The t(11;16)(q23;p13) translocation in myelodysplastic syndrome fuses the MLL gene to the CBP gene. Blood 1997;89:3945-3950.

191. Kreuziger LM, Porcher JC, Ketterling RP, Steensma DP. An MLL-SEPT9 fusion and t(11;17)(q23;q25) associated with de novo myelodysplastic syndrome. Leuk.Res. 2007;31:1145-1148.

192. Stevens-Kroef MJ, Schoenmakers EF, van KM et al. Identification of truncated RUNX1 and RUNX1-PRDM16 fusion transcripts in a case of t(1;21)(p36;q22)-positive therapy-related AML. Leukemia 2006;20:1187-1189.

193. Mochizuki N, Shimizu S, Nagasawa T et al. A novel gene, MEL1, mapped to 1p36.3 is highly homologous to the MDS1/EVI1 gene and is transcriptionally activated in t(1;3)(p36;q21)-positive leukemia cells. Blood 2000;96:3209-3214.

194. Ahuja HG, Felix CA, Aplan PD. The t(11;20)(p15;q11) chromosomal translocation associated with therapy-related myelodysplastic syndrome results in an NUP98-TOP1 fusion. Blood 1999;94:3258-3261.

195. Hatano Y, Miura I, Nakamura T et al. Molecular heterogeneity of the NUP98/HOXA9 fusion transcript in myelodysplastic syndromes associated with t(7;11)(p15;p15). Br.J.Haematol. 1999;107:600-604.

196. Lyons J, Janssen JW, Bartram C, Layton M, Mufti GJ. Mutation of Ki-ras and N-ras oncogenes in myelodysplastic syndromes. Blood 1988;71:1707-1712.

197. Padua RA, Carter G, Hughes D et al. RAS mutations in myelodysplasia detected by amplification, oligonucleotide hybridization, and transformation. Leukemia 1988;2:503-510.

198. Ahuja HG, Foti A, Bar-Eli M, Cline MJ. The pattern of mutational involvement of RAS genes in human hematologic malignancies determined by DNA amplification and direct sequencing. Blood 1990;75:1684-1690.

199. Nakagawa T, Saitoh S, Imoto S et al. Multiple point mutation of N-ras and K-ras oncogenes in myelodysplastic syndrome and acute myelogenous leukemia. Oncology 1992;49:114-122.

200. van KH, de PC, Verlaan-de VM et al. Longitudinal analysis of point mutations of the N-ras proto-oncogene in patients with myelodysplasia using archived blood smears. Blood 1992;79:1266-1270.

201. Horiike S, Misawa S, Nakai H et al. N-ras mutation and karyotypic evolution are closely associated with leukemic transformation in myelodysplastic syndrome. Leukemia 1994;8:1331-1336.

202. Constantinidou M, Chalevelakis G, Economopoulos T et al. Codon 12 ras mutations in patients with myelodysplastic syndrome: incidence and prognostic value. Ann.Hematol. 1997;74:11-14.

203. Misawa S, Horiike S, Kaneko H, Kashima K. Genetic aberrations in the development and subsequent progression of myelodysplastic syndrome. Leukemia 1997;11 Suppl 3:533-535.

204. Padua RA, Guinn BA, Al-Sabah AI et al. RAS, FMS and p53 mutations and poor clinical outcome in myelodysplasias: a 10-year follow-up. Leukemia 1998;12:887-892.

205. Lu D, Nounou R, Beran M et al. The prognostic significance of bone marrow levels of neurofibromatosis-1 protein and ras oncogene mutations in patients with acute myeloid leukemia and myelodysplastic syndrome. Cancer 2003;97:441-449.

206. Paquette RL, Landaw EM, Pierre RV et al. N-ras mutations are associated with poor prognosis and increased risk of leukemia in myelodysplastic syndrome. Blood 1993;82:590-599.

207. Georgiou G, Karali V, Zouvelou C et al. Serial determination of FLT3 mutations in myelodysplastic syndrome patients at diagnosis, follow up or acute myeloid leukaemia transformation: incidence and their prognostic significance. Br.J.Haematol. 2006;134:302-306.

208. Yamamoto Y, Kiyoi H, Nakano Y et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood 2001;97:2434-2439.

209. Horiike S, Yokota S, Nakao M et al. Tandem duplications of the FLT3 receptor gene are associated with leukemic transformation of myelodysplasia. Leukemia 1997;11:1442-1446.

210. Yokota S, Kiyoi H, Nakao M et al. Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. Leukemia 1997;11:1605-1609.

211. Bacher U, Haferlach T, Kern W, Haferlach C, Schnittger S. A comparative study of molecular mutations in 381 patients with myelodysplastic syndrome and in 4130 patients with acute myeloid leukemia. Haematologica 2007;92:744-752.

212. Johan MF, Bowen DT, Frew ME et al. Mutations in PTPN11 are uncommon in adult myelodysplastic syndromes and acute myeloid leukaemia. Br.J.Haematol. 2004;124:843-844.

213. Loh ML, Martinelli S, Cordeddu V et al. Acquired PTPN11 mutations occur rarely in adult patients with myelodysplastic syndromes and chronic myelomonocytic leukemia. Leuk.Res. 2005;29:459-462.

214. Watkins F, Fidler C, Boultwood J, Wainscoat JS. Mutations in PTPN11 are rare in adult myelodysplastic syndromes and acute myeloid leukemia. Am.J.Hematol. 2004;76:417.

215. Steensma DP, Dewald GW, Lasho TL et al. The JAK2 V617F activating tyrosine kinase mutation is an infrequent event in both "atypical" myeloproliferative disorders and myelodysplastic syndromes. Blood 2005;106:1207-1209.

216. Chen CY, Lin LI, Tang JL et al. Acquisition of JAK2, PTPN11, and RAS mutations during disease progression in primary myelodysplastic syndrome. Leukemia 2006;20:1155-1158.

217. Ridge SA, Worwood M, Oscier D, Jacobs A, Padua RA. FMS mutations in myelodysplastic, leukemic, and normal subjects. Proc.Natl.Acad.Sci.U.S.A 1990;87:1377-1380.

218. Lorenzo F, Nishii K, Monma F et al. Mutational analysis of the KIT gene in myelodysplastic syndrome (MDS) and MDS-derived leukemia. Leuk.Res. 2006;30:1235-1239.

219. Fritsche-Polanz R, Jordan JH, Feix A et al. Mutation analysis of C-KIT in patients with myelodysplastic syndromes without mastocytosis and cases of systemic mastocytosis. Br.J.Haematol. 2001;113:357-364.

220. Wattel E, Preudhomme C, Hecquet B et al. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. Blood 1994;84:3148-3157.

221. Adamson DJ, Dawson AA, Bennett B, King DJ, Haites NE. p53 mutation in the myelodysplastic syndromes. Br.J.Haematol. 1995;89:61-66.

222. Kaneko H, Misawa S, Horiike S, Nakai H, Kashima K. TP53 mutations emerge at early phase of myelodysplastic syndrome and are associated with complex chromosomal abnormalities. Blood 1995;85:2189-2193.

223. Jonveaux P, Fenaux P, Quiquandon I et al. Mutations in the p53 gene in myelodysplastic syndromes. Oncogene 1991;6:2243-2247.

224. Ludwig L, Schulz AS, Janssen JW, Grunewald K, Bartram CR. P53 mutations in myelodysplastic syndromes. Leukemia 1992;6:1302-1304.

225. Sugimoto K, Hirano N, Toyoshima H et al. Mutations of the p53 gene in myelodysplastic syndrome (MDS) and MDS-derived leukemia. Blood 1993;81:3022-3026.

226. Kaneko H, Horiike S, Nakao M et al. Mutation of CDKN2 is infrequently detected in myelodysplastic syndrome. Leukemia 1997;11:1391-1392.

227. Papageorgiou SG, Pappa V, Papageorgiou E et al. Absence of p16 and p27 gene rearrangements and mutations in de novo myelodysplastic syndromes. Eur.J.Haematol. 2005;75:193-198.

228. Nakamaki T, Bartram C, Seriu T et al. Molecular analysis of the cyclin-dependent kinase inhibitor genes, p15, p16, p18 and p19 in the myelodysplastic syndromes. Leuk.Res. 1997;21:235-240.

229. Nakao M, Horiike S, Fukushima-Nakase Y et al. Novel loss-of-function mutations of the haematopoiesis-related transcription factor, acute myeloid leukaemia 1/runt-related transcription factor 1, detected in acute myeloblastic leukaemia and myelodysplastic syndrome. Br.J.Haematol. 2004;125:709-719.

230. Fuchs O, Provaznikova D, Kocova M et al. CEBPA polymorphisms and mutations in patients with acute myeloid leukemia, myelodysplastic syndrome, multiple myeloma and non-Hodgkin's lymphoma. Blood Cells Mol.Dis. 2008;40:401-405.

231. Christiansen DH, Andersen MK, Desta F, Pedersen-Bjergaard J. Mutations of genes in the receptor tyrosine kinase (RTK)/RAS-BRAF signal transduction pathway in therapy-related myelodysplasia and acute myeloid leukemia. Leukemia 2005;19:2232-2240.

232. Mori N, Okada M, Motoji T, Mizoguchi H. Mutation of the WT1 gene in myelodysplastic syndrome and acute myeloid leukaemia post myelodysplastic syndrome. Br.J.Haematol. 1999;105:844-845.

233. Zhang Y, Zhang M, Yang L, Xiao Z. NPM1 mutations in myelodysplastic syndromes and acute myeloid leukemia with normal karyotype. Leuk.Res. 2007;31:109-111.

234. Makishima H, Cazzolli H, Szpurka H et al. Mutations of e3 ubiquitin ligase cbl family members constitute a novel common pathogenic lesion in myeloid malignancies. J.Clin.Oncol. 2009;27:6109-6116.

235. Andrulis M, Capper D, Luft T et al. Detection of isocitrate dehydrogenase 1 mutation R132H in myelodysplastic syndrome by mutation-specific antibody and direct sequencing. Leuk.Res. 2010;34:1091-1093.

236. Kosmider O, Gelsi-Boyer V, Slama L et al. Mutations of IDH1 and IDH2 genes in early and accelerated phases of myelodysplastic syndromes and MDS/myeloproliferative neoplasms. Leukemia 2010;24:1094-1096.

237. Thol F, Weissinger EM, Krauter J et al. IDH1 mutations in patients with myelodysplastic syndromes are associated with an unfavorable prognosis. Haematologica 2010;95:1668-1674.

238. Papaemmanuil E, Cazzola M, Boultwood J et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. N.Engl.J.Med. 2011;365:1384-1395.

239. Yoshida K, Sanada M, Shiraishi Y et al. Frequent pathway mutations of splicing machinery in myelodysplasia. Nature 2011;478:64-69.

Chapter 2

Apoptosis-related gene expression profiling in hematopoietic cell fractions of MDS patients

Submitted

SMC Langemeijer, R Knops, C Gilissen, R Woestenenk, T de Witte, BA van der Reijden, JH Jansen

Abstract

Although the vast majority of patients with a myelodysplastic syndrome (MDS) suffer from cytopenias, the bone marrow is usually normocellular or hypercellular. Apoptosis of hematopoietic cells in the bone marrow has been implicated in this phenomenon. However, in MDS it remains only partially elucidated which genes are involved in this process and which hematopoietic cells are mainly affected. We have employed sensitive real-time PCR technology to study a large set of apoptosis-related genes and gene families in the immature CD34+ and the differentiating erythroid and monomyeloid bone marrow cells. Several genes were found to be differentially expressed between patients and controls in more than one cell fraction, most importantly the *BIK (BCL2-interacting killer)* gene. Although different patient groups, based on the IPSS scoring system, and healthy controls could not be distinguished based on the overall expression pattern of these apoptosis-related genes, the differential expression of specific genes in MDS patients and controls may contribute to changes in the cell's sensitivity to cell death and thus increased apoptosis.

Introduction

Myelodysplastic syndromes (MDS) represent a heterogeneous group of malignant hematopoietic disorders that are characterized by dysplasia in the myeloid, megakaryocytic and/or erythroid cell lineages. The clinical course of MDS is highly variable. Whereas some patients suffer from smoldering cytopenias, others rapidly develop a more aggressive disease eventually resulting in acute myeloid leukemia. The WHO classification and International Prognostic Scoring system (IPSS) recognize the heterogeneity of MDS and divide patients into subgroups based on characteristics such as the amount of blasts in the bone marrow and the number of cytopenias.

Although cytopenias are present in the peripheral blood of the vast majority of MDS patients, the bone marrow is usually normocellular or hypercellular. Apoptosis of hematopoietic cells in the bone marrow has been implicated in this phenomenon. Several studies have shown signs of increased apoptosis in MDS bone marrow using techniques such as *in situ end labeling* (ISEL) of fragmented DNA/ TUNEL assay [1], electron microscopy [2], flowcytometry using annexin V staining [3, 4] and measurement of mitochondrial membrane potential [4]. The percentage of cells affected by apoptosis differs between studies, possibly due to the use of different techniques and the heterogeneity of the clinical samples studied. In most, but not all studies apoptosis markers are particularly elevated in the more indolent cases of MDS, whereas apoptosis is reduced or at normal levels in the more aggressive cases. This led to the hypothesis that apoptosis is initially increased in MDS due to either primary defects of the apoptotic pathway or in response to oncogenic stress. During the advanced stages of MDS, this response may be lost as result of further transformation of the malignant cells. The presence of apoptosis has been studied in whole bone marrow and in several bone marrow fractions, such as CD34+ cells. Depending on the study, increased apoptosis parameters were found in the CD34+ cell fraction of all MDS patients[4, 5] or only in patients with the more indolent phenotype[3]. Others described that apoptosis is mainly increased in the more committed myeloid, erythroid and/ or megakaryocytic lineages[2, 6].

Apoptosis is a tightly regulated process that involves many proteins. The differential regulation of some of these proteins, such as Fas[7, 8], FLIP [9, 10], BCL-2 and BCL-2-related proteins[3, 11-14], TNF proteins and their receptors [15-17], IAPs [18, 19] and caspases [14, 20] has been the subject of studies in MDS patients. Most of these studies focused on only one or a few apoptosis-related genes and were performed in either whole bone marrow or the CD34+ cell fraction.

Based on the results of these previous studies, we expected that different patterns of apoptosis-related gene expression can be distinguished in healthy hematopoietic cells and those of low-risk and high-risk MDS patients. To gain insight into these expression patterns, we have employed sensitive real-time PCR technology to study 93 apoptosis-related genes and gene families in both CD34+ immature hematopoietic cells as well as differentiated erythroid and monomyeloid cells.

Results

Isolation of hematopoietic cell fractions

Hematopoietic cell fractions were isolated from the bone marrow of 23 patients and 10 healthy controls (**Supplementary Table 1**). Three different hematopoietic cell fractions were sorted, CD34+ cells (immature hematopoietic cells), CD71+ erythroid precursors and CD13/33+ mono-myeloid precursors. According to the IPSS score 4 low , 11 int-1, 4 int-2 and 4 high risk patients were included. In most cases we were able to isolate all three subfractions from the same patient (**Supplementary Table 1**). As a control, the expression of several genes related to erythroid differentiation (*TFRC, GYPA, GYPB, GYPC, SPTB, SPTA1, EBP41, EBP42* and *HBB)* was measured in all fractions and shown to be highly upregulated in CD71+ cells (data not shown).

Unsupervised cluster analysis separates different bone marrow fractions but does not differentiate MDS patients and controls

Unsupervised hierarchical cluster analysis was performed based on the expression of 93 apoptosis-related genes. These included members of the TNF (Receptor) Family, BCL-2 Family, IAPs, Caspases, CARD Family, TRAF family and others (**Supplementary Table 2**). The analysis showed a clear clustering of the three hematopoietic cell fractions, indicating different patterns of apoptosis-related gene activity in these cell fractions (**Figure 1)**. Unexpectedly, within each cell fraction, patients and healthy controls could not be distinguished based on the global expression pattern of these apoptosis-related genes (**Figure 1**). Also low-risk MDS and high-risk MDS patient groups could not be distinguished using this approach.

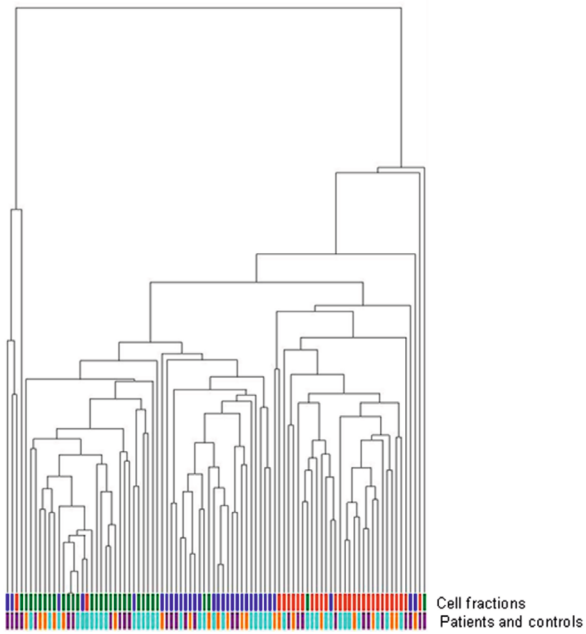


Figure 1: Unsupervised hierarchical cluster analysis of all samples based on apoptosis-related gene expression profile.
Cell fractions are shown in the first column: CD34+ cells (blue), CD71+ cells (green), CD13/33+ cells (red). The second column shows patients and controls: controls (purple), low risk MDS (light blue), high risk MDS (orange)

Differential expression of apoptosis-related genes in MDS patients and controls

Analysis of individual genes revealed several genes that were differentially expressed between patients and controls in more than one cell fraction: *BIK, TNFRSF19* and *TNFRSF7* (CD34+ and CD71+ fractions) and *MDM2* (CD71+ and CD13/33+ fractions). (**Table 1, Figure 2**) Of these genes, only the pro-apoptotic *BIK* gene showed a significantly increased expression after correction of p-values for multiple testing. The analyses were repeated after the MDS patients were divided into low-risk and high-risk groups. As was the case for the comparison between all patients and controls, differential expression was detected of *TNFRSF19, RALBP1* and *TNFRSF7* in the CD34+ fraction, *BIK, DAPK1, BNIPL, TNFSRF10A,* and *CARD9* in the CD71+ group and *TNFSRF13B* and *MDM2* in the CD13/33+ fraction. In addition, several other genes mainly showed differences in expression between the low-risk and high-risk MDS patients and not between patients and controls (**Supplementary Table 3**). The apoptosis-related genes *TNFRSF4, BIK* and *TNFRSF13B* showed the most prominent differences in expression between the different patient groups and controls. Although several pro-apoptotic genes showed increased expression in low-risk MDS patients and anti-apoptotic genes reduced expression, no clear correlation was seen between the known effect of the genes studied (pro- or anti-apoptotic) and the MDS subtype.

Table 1A: Differentially expressed genes in the CD34+ cell fraction in MDS patients versus controls

Gene	p-value	effect on apoptosis	Ratio of median expression in patients/ controls
TNFRSF19	0.006	pro	0.2
TNFRSF7	0.013	pro	0.1
BIK	0.016	pro	0.6
RIPK2	0.020	pro	1.5
CARD11	0.035	pro	0.6
RALBP1	0.047	anti	2.9

Table 1B: Differentially expressed genes in the CD71+ cell fraction in MDS patients versus controls

Gene	p-value	effect on apoptosis	Ratio of median expression in patients/ controls
BIK	0.001*	pro	203
BNIPL	0.004	pro	0.1
DAPK1	0.020	pro	3.6
BBC3	0.015	pro	3.0
EDA2R	0.015	pro	49
TNFRSF10A	0.015	pro	3.9
CARD9	0.018	pro	1.6
CASP9	0.018	pro	7.0
DAPK1	0.012	pro	3.3
MDM2	0.020	anti	1.8
TNFSRF19	0.020	pro	0.2
TNFRSF7	0.022	pro	0.2
TNFRSF10B	0.028	pro	6.2
BCL2L10	0.032	anti	2.1
BAG3	0.035	anti	3.1
TNFRSF10D	0.044	anti	2.9

Table 1C: Differentially expressed genes in the CD13/33+ cell fraction in MDS patients versus controls

Gene	p-value	effect on apoptosis	Ratio of median expression in patients/ controls
MDM2	0.004	anti	1.7
TNFRSF13B	0.005	-	0.01
FADD	0.035	pro	1.7

**indicates significant difference ($p<0.05$) using p-value for multiple testing*

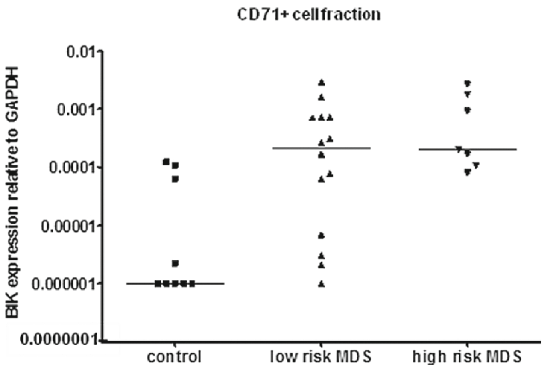


Figure 2: Expression of BIK in MDS patients and controls
BIK expression in 'low risk' patients, 'high risk' patients and controls. The expression of BIK is depicted relative to the housekeeping gene GAPDH. Median level of expression is indicated.

Discussion

Up- or downregulation of various apoptosis-related genes in MDS has been shown in the past. We have studied the expression of a large set of genes and gene families that have been associated with apoptosis in three different FACS isolated hematopoietic cell fractions. Using sensitive real-time PCR analysis followed by unsupervised clustering analysis of the measured gene expression levels, we were able to distinguish the CD34+, CD71+ and CD13/33+ cell fractions. Unexpectedly, global patterns of gene expression could not distinguish healthy controls and low risk and high risk MDS patients, but analysis of the expression of single genes identified a limited number of genes that were differentially expressed between these groups. Deregulation of the expression of some of these genes has been implicated in MDS before. Increased expression of *TNFRSF10A (TRAIL-R1)* in MDS has been described, though not specifically in the CD71+ population [16]. Differential expression of other genes, such as *TP53* and *BCL2*, that have previously been implicated in MDS, could not be confirmed. Differences between our study and those of others could be due to the bone marrow fractions studied and the selection of patients. The *BIK* (BCL-2 interacting killer) gene showed the most prominent differences in expression between MDS patients and controls. *BIK* is thought to mediate apoptosis signaling to mitochondria. Although mutations of *BIK* have been found in B-cell lymphomas [21], little is known about the role of *BIK* in myeloid malignancies. In a study examining gene expression in acute myeloid leukemia, *BIK* was found to be upregulated in AML samples compared to controls [22]. However, the differences in expression were dependent on the cytogenetic abnormalities of the patients.

We conclude that, although apoptosis has been found to be disturbed in MDS, the overall expression pattern of a large number of apoptosis-related genes is not grossly disturbed in MDS. When single genes were analyzed, the expression of *BIK* appeared to be the most prominently differentially expressed gene. Further functional studies into the possible role of *BIK* in MDS are therefore warranted.

Methods

Patients and controls

Bone marrow from MDS patients (n=23) and healthy controls (n=10) was collected after informed consent. Patients diagnosed at the Radboud University Nijmegen Medical Centre belonging to all different WHO and IPSS categories were included. Patient characteristics are shown in **Supplementary Table 1**. Mononuclear cells were isolated from the bone marrow by density gradient centrifugation using Ficoll 1.077 g/mL (Pharmacia Biotech, Uppsala, Sweden) and viably frozen in liquid nitrogen until further use.

Cell sorting and RNA isolation

After thawing, hematopoietic cell subtypes were isolated by fluorescence activated cell sorting (FACS) using monoclonal antibodies directed against CD45 and the side scatter profile. In addition, monoclonal antibodies directed against CD34, CD13, CD33 and CD71 were used. Gating on forward and side scatter was used to exclude dead cells and debris. RNA was isolated from three subfractions, the CD34+ stem/progenitor cells, the CD13/33+ mono/myeloid cells and the CD71+ erythroid cells.

Gene expression analysis

In a first set of patients and controls, the expression of 180 different apoptosis-related genes was measured simultaneously by real-time PCR employing a 384 Microfluidic Card (Applied Biosystems). The genes measured and the corresponding assay numbers are shown in **Supplementary Table 2**. cDNA obtained from the CD34+ (patients n=13, controls n=5), CD71+ (patients n=12, controls n=4) and CD13/33+ (patients n=11, controls n= 5) cell fractions was analyzed. Based on the expression pattern of these genes, a selection of 93 genes was used to perform a similar expression analysis in an additional group of CD34+ (patients n=8, controls n=5), CD71+ (patients n=9, controls n=5) and CD13/33+ cells (patients n=8, controls n=5). The relative expression of the apoptosis-related genes was calculated by measuring the housekeeping gene *GAPDH*. Relative expression values less than $1x10^{-6}$ were set to $1x10^{-6}$ to avoid bias introduced by including values reaching the limit of detection of the real-time PCR assay. In addition to the apoptosis-related genes, the expression of several genes implicated in erythroid differentiation (*TFRC, GYPA, GYPB, GYPC, SPTB, SPTA1, EBP41, EBP42* and *HBB*) was determined to check whether these genes indeed showed highest expression in the CD71+ cell fraction. The erythroid genes were only used as controls and not included in subsequent statistical analyses.

Statistical analysis

Microfluidic card log-scale values were imported into Partek (Partek Genomic Suite software, version 6.5; Partek Inc., St. Louis, MO). Principle Component Analysis (PCA) showed a clear distinction between the three different tissues and thus the dataset was split accordingly into three separate groups. In each group expression differences between donors and patients was calculated using the non-parametric Mann-Whitney U-test. For statistical analysis, patients from the IPSS low and int-1 risk groups were combined in a 'low risk MDS' group and patients from the IPSS int-2 and high-risk groups in a 'high-risk MDS' group. Corresponding P-values were corrected for multiple testing by random permutations. The Kruskall-Wallis-test was used to identify differences between the 'low risk MDS', 'high-risk MDS' and control groups. In case of a p-value< 0.05, posthoc analysis was perfomed using non-parametric Mann-Whitney U test.

Reference List

1. Tsoplou,P. et al. Apoptosis in patients with myelodysplastic syndromes: differential involvement of marrow cells in 'good' versus 'poor' prognosis patients and correlation with apoptosis-related genes. Leukemia 13, 1554-1563 (1999).
2. Bogdanovic,A.D. et al. Incidence and role of apoptosis in myelodysplastic syndrome: morphological and ultrastructural assessment. Leukemia 11, 656-659 (1997).
3. Parker,J.E. et al. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. Blood 96, 3932-3938 (2000).
4. Huh,Y.O. et al. More cell death in refractory anemia with excess blasts in transformation than in acute myeloid leukemia. Leukemia 16, 2249-2252 (2002).
5. Lin,C.W. et al. Proliferation and apoptosis in acute and chronic leukemias and myelodysplastic syndrome. Leuk. Res. 26, 551-559 (2002).
6. Raza,A. et al. Simultaneous assessment of cell kinetics and programmed cell death in bone marrow biopsies of myelodysplastics reveals extensive apoptosis as the probable basis for ineffective hematopoiesis. Am. J. Hematol. 48, 143-154 (1995).
7. Bouscary,D. et al. Fas/Apo-1 (CD95) expression and apoptosis in patients with myelodysplastic syndromes. Leukemia 11, 839-845 (1997).
8. Claessens,Y.E. et al. In vitro proliferation and differentiation of erythroid progenitors from patients with myelodysplastic syndromes: evidence for Fas-dependent apoptosis. Blood 99, 1594-1601 (2002).
9. Benesch,M., Platzbecker,U., Ward,J., Deeg,H.J., & Leisenring,W. Expression of FLIP(Long) and FLIP(Short) in bone marrow mononuclear and CD34+ cells in patients with myelodysplastic syndrome: correlation with apoptosis. Leukemia 17, 2460-2466 (2003).
10. de Melo,C.P. et al. Reduced expression of FLIP SHORT in bone marrow of low risk myelodysplastic syndrome. Leuk. Res. 31, 853-857 (2007).
11. Rajapaksa,R., Ginzton,N., Rott,L.S., & Greenberg,P.L. Altered oncoprotein expression and apoptosis in myelodysplastic syndrome marrow cells. Blood 88, 4275-4287 (1996).
12. Parker,J.E. et al. 'Low-risk' myelodysplastic syndrome is associated with excessive apoptosis and an increased ratio of pro- versus anti-apoptotic bcl-2-related proteins. Br. J. Haematol. 103, 1075-1082 (1998).
13. Suarez,L. et al. Expression of APO2.7, bcl-2 and bax apoptosis-associated proteins in CD34- bone marrow cell compartments from patients with myelodysplastic syndromes. Leukemia 18, 1311-1313 (2004).
14. Economopoulou,C. et al. Analysis of apoptosis regulatory genes expression in the bone marrow (BM) of adult de novo myelodysplastic syndromes (MDS). Leuk. Res. 32, 61-69 (2008).
15. Kitagawa,M. et al. Overexpression of tumor necrosis factor (TNF)-alpha and interferon (IFN)-gamma by bone marrow cells from patients with myelodysplastic syndromes. Leukemia 11, 2049-2054 (1997).
16. Zang,D.Y., Goodwin,R.G., Loken,M.R., Bryant,E., & Deeg,H.J. Expression of tumor necrosis factor-related apoptosis-inducing ligand, Apo2L, and its receptors in myelodysplastic syndrome: effects on in vitro hemopoiesis. Blood 98, 3058-3065 (2001).
17. Sawanobori,M. et al. Expression of TNF receptors and related signaling molecules in the bone marrow from patients with myelodysplastic syndromes. Leuk. Res. 27, 583-591 (2003).
18. Gianelli,U. et al. Survivin expression in "low-risk" and "high-risk" myelodysplastic syndromes. Ann. Hematol. 86, 185-189 (2007).

19. Yamamoto,K. et al. Expression of IAP family proteins in myelodysplastic syndromes transforming to overt leukemia. Leuk. Res. 28, 1203-1211 (2004).

20. Boudard,D. et al. Expression and activity of caspases 1 and 3 in myelodysplastic syndromes. Leukemia 14, 2045-2051 (2000).

21. Arena,V., Martini,M., Luongo,M., Capelli,A., & Larocca,L.M. Mutations of the BIK gene in human peripheral B-cell lymphomas. Genes Chromosomes. Cancer 38, 91-96 (2003).

22. Stirewalt,D.L. et al. Identification of genes with abnormal expression changes in acute myeloid leukemia. Genes Chromosomes. Cancer 47, 8-20 (2008).

Supplementary information

Supplementary Table 1: Patients and controls

No.	FAB	WHO	IPSS	Karyotype	Blasts in BM	Hb (mmol/l)	Granulocytes (x109/l)	Cell fractions analyzed
patient 1	RA	RCMD	low	normal	3	5.7	2.1	CD34, CD13/33
patient 2	RA	RCMD	low	normal	1	7.0	1.9	CD34, CD71, CD13/33
patient 3	RA	RCMD	low	normal	1	3.8	5.8	CD34, CD71, CD13/33
patient 4	RARS	RCMD-RS	low	normal	1	2.4	2.1	CD34, CD71, CD13/33
patient 5	RA	RA	int-1	46,XY, -20, +mar [10]	3	6.2	2.6	CD34, CD71, CD13/33
patient 6	RARS	RARS	int-1	normal	2	6.0	3.6	CD34, CD71
patient 7	RARS	RARS	int-1	47,XY,+8[4]/46,XY[6]	2	5.6	2.4	CD34, CD71, CD13/33
patient 8	RARS	RCMD-RS	int-1	normal	1	3.4	0.7	CD71
patient 9	RAEB	RAEB-1	int-1	normal	6	6.0	0.2	CD34, CD71, CD13/33
patient 10	RAEB	RAEB-1	int-1	normal	9	7.5	4.5	CD34, CD71, CD13/33
patient 11	RAEB	RAEB-1	int-1	47,XX,+8[7]/46,XX[3]	6	6.5	28.1	CD34, CD71, CD13/33
patient 12	RAEB	RAEB-1	int-1	normal	8	7.7	0.5	CD34, CD71, CD13/33
patient 13	RAEB	RAEB-1	int-1	normal	5	5.6	1.9	CD34, CD71
patient 14	RAEB	RAEB-1	int-1	normal	6	4.0	1.0	CD34, CD71, CD13/33
patient 15	RAEB	RAEB-1	int-1	normal	5	3.8	1.7	CD71, CD13/33
patient 16	RAEB	RAEB-2	int-2	normal	4	6.6	2.5	CD34, CD71, CD13/33
patient 17	RAEB-t	RAEB-2	int-2	normaal	12	5.9	21.6	CD34, CD71, CD13/33
patient 18	RAEB-t	RAEB-2	int-2	complex	10	6.6	12.1	CD34, CD71, CD13/33
patient 19	RAEB-t	AML	int-2	normal	21	6.7	2.5	CD34, CD71, CD13/33
patient 20	RAEB-t	RAEB-2	high	complex	11	4.0	0.6	CD34, CD71
patient 21	RAEB	RAEB-2	high	47,XY, +11 [5] / 46,XY [15]	12	6.2	1.0	CD34, CD13/33
patient 22	RAEB-t	AML	high	normal	22	4.8	0.6	CD34, CD71, CD13/33
patient 23	RAEB-t	AML	high	complex	22	6.3	1.3	CD34, CD71, CD13/33
controls 1-9								CD34, CD71, CD13/33
control 10								CD34, CD13/33

Supplementary Table 2: Genes and assay numbers

Gene	NM number	Assay number	Assay
BAX	NM_138761.2	Hs00180269_m1	1,2
BBC3	NM_014417.2	Hs00248075_m1	1,2
BCL2A1	NM_004049.2	Hs00187845_m1	1,2
BCL2	NM_000633.2	Hs00153350_m1	1,2
BCL2L10	NM_020396.2	Hs00368095_m1	1,2
BCL2L11	NM_138621.2	Hs00197982_m1	1,2
BCL2L12	NM_138639.1	Hs00369327_m1	1,2
BCL2L13	NM_015367	Hs00209789_m1	1
BCL2L14	NM_030766.1	Hs00373302_m1	1,2
BCL2L1	NM_001191	Hs00169141_m1	1,2
BCL2L1	NM_138578.1	Hs00236329_m1	1,2
BCL2L2	NM_004050	Hs00187848_m1	1
BID	NM_197966.1	Hs00609630_m1	1,2
BIK	NM_001197.3	Hs00154189_m1	1,2
BLK	NM_001715.2	Hs00176441_m1	1,2
BNIP1	NM_001205.1	Hs00241824_m1	1,2
BNIP2	NM_004330.1	Hs00188939_m1	1,2
BNIPL	NM_138278.2	Hs00414503_m1	1,2
BOK	NM_032515.3	Hs00261296_m1	1,2
HRK	NM_003806.1	Hs00705213_s1	1,2
BAD	NM_032989	Hs00188930_m1	1
BAG1	NM_004323	Hs00185390_m1	1
BAG2	NM_004282.2	Hs00188716_m1	1,2
BAG3	NM_004281.3	Hs00188713_m1	1,2
BAG4	NM_004874	Hs00362193_m1	1
BAG5	NM_004873	Hs00191644_m1	1
BAK1	NM_001188	Hs00832876_g1	1
BCL10	NM_003921	Hs00184839_m1	1
BCL3	NM_005178	Hs00180403_m1	1
BCLAF1	NM_014739	Hs00602256_m1	1
BECN1	NM_003766	Hs00186838_m1	1
BMF	NM_033503	Hs00372938_m1	1
BNIP3L	NM_004331	Hs00188949_m1	1
C19orf6	NM_033420	Hs00364301_m1	1

Supplementary Table 2: Genes and assay numbers (vervolg)

Gene	NM number	Assay number	Assay
MCL1	NM_021960	Hs00172036_m1	1
MCL1	NM_182763	Hs00766187_m1	1
PMAIP1	NM_021127	Hs00560402_m1	1
CARD10	NM_014550.3	Hs00367225_m1	1,2
CARD11	NM_032415.2	Hs00260906_m1	1,2
CARD12	NM_021209.3	Hs00368367_m1	1,2
CARD14	NM_024110	Hs00364499_m1	1
CARD15	NM_022162.1	Hs00223394_m1	1,2
CARD4	NM_006092	Hs00196075_m1	1
CARD6	NM_032587.2	Hs00261581_m1	1,2
CARD8	NM_014959.1	Hs00209095_m1	1,2
CARD9	NM_052813.2	Hs00364485_m1	1,2
NOL3	NM_003946.3	Hs00358724_g1	1,2
RIPK2	NM_003821.5	Hs00169419_m1	1,2
APAF1	NM_181861	Hs00559441_m1	1
C10orf97	NM_024948	Hs00227894_m1	1
C9orf89	NM_032310	Hs00260439_m1	1
COPl	NM_052889	Hs00430993_m1	1
NALP1	NM_033004	Hs00248187_m1	1
PYCARD	NM_145183	Hs00203118_m1	1
CASP3	NM_032991.2	Hs00234387_m1	1,2
CASP5	NM_004347.1	Hs00237061_m1	1,2
CASP6	NM_032992.2	Hs00154250_m1	1,2
CASP7	NM_033338.4	Hs00169152_m1	1,2
CASP8AP2	NM_012115.2	Hs00201640_m1	1,2
CASP8	NM_033356.2	Hs00154256_m1	1,2
CASP9	NM_032996.1	Hs00154260_m1	1,2
PACAP	NM_016459.3	Hs00414907_m1	1,2
CASP10	NM_32974	Hs00154268_m1	1
CASP14	NM_012114	Hs00201637_m1	1
CASP1	NM_03329	Hs00169146_m1	1
CASP2	NM_032982	Hs00154242_m1	1
CASP4	NM_001225.3	Hs00426677_m1	1
CASP8	NM_033356.2	Hs00236278_m1	1
CIDEC	NM_022094.2	Hs00535723_m1	1,2
CIDEA	NM_198289	Hs00154455_m1	1

Gene	NM number	Assay number	Assay
CIDEB	NM_014430	Hs00205339_m1	1
CRADD	NM_003805.3	Hs00187009_m1	1,2
EDARADD	NM_080738.2	Hs00369830_m1	1,2
FADD	NM_003824.2	Hs00538709_m1	1,2
LRDD	NM_018494	Hs00388035_m1	1
MADD	NM_130470	Hs00366249_m1	1
TRADD	NM_003789	Hs00182558_m1	1
DEDD2	NM_032998	Hs00370206_m1	1
DEDD	NM_133328	Hs00172768_m1	1
BIRC3	NM_182962.1	Hs00154109_m1	1,2
BIRC7	NM_139317.1	Hs00223384_m1	1,2
BIRC8	NM_033341.3	Hs00364262_s1	1,2
BIRC1	NM_004536	Hs00244967_m1	1
BIRC2	NM_001166	Hs00357350_m1	1
BIRC4BP	NM_017523	Hs00213882_m1	1
BIRC4	NM_001167	Hs00236913_m1	1
BIRC5	NM_001012271	Hs00153353_m1	1
BIRC6	NM_016252	Hs00212288_m1	1
ABL2	NM_005158	Hs00246861_m1	1,2
ABL2	NM_007314	Hs00270858_m1	1,2
ATM	NM_138292.3	Hs00175892_m1	1,2
CHEK1	NM_001274.2	Hs00176236_m1	1,2
CHEK2	NM_001005735.1	Hs00200485_m1	1,2
DUSP2	NM_004418.2	Hs00358879_m1	1,2
GADD45A	NM_001924.2	Hs00169255_m1	1,2
MDM1	NM_017440.2	Hs00220015_m1	1,2
MDM2	NM_002392.2	Hs00242813_m1	1,2
PARK2	NM_013987.1	Hs00247755_m1	1,2
TP53BP1	NM_005657.1	Hs00152943_m1	1,2
TP53	NM_000546.2	Hs00153349_m1	1,2
TP73	NM_005427.1	Hs00232088_m1	1,2
TP73L	NM_003722.3	Hs00186613_m1	1,2
ABL1	NM_005157	Hs00245445_m1	1
AKT1	NM_001014431	Hs00178289_m1	1
GADD45B	NM_015675	Hs00169587_m1	1
P53AIP1	NM_022112	Hs00223141_m1	1

Supplementary Table 2: Genes and assay numbers (vervolg)

Gene	NM number	Assay number	Assay
PARC	NM_015089	Hs00292746_m1	1
PERP	NM_022121	Hs00751717_s1	1
PIN1	NM_006221	Hs00749260_s1	1
PPM1D	NM_003620	Hs00186230_m1	1
PTEN	NM_000314	Hs00829813_s1	1
RCHY1	NM_001008925	Hs00295839_m1	1
RFWD2	NM_022457	Hs00375437_m1	1
RPA3	NM_002947	Hs00366098_m1	1
TP53BP2	NM_005657.1	Hs00610488_m1	1
PDCD1	NM_005018.1	Hs00169472_m1	1,2
PDCD4	NM_145341.2	Hs00377253_m1	1,2
PDCD5	NM_004708.2	Hs00270435_m1	1,2
FAS	NM_152871.1	Hs00531110_m1	1,2
TNFRSF10A	NM_003844.2	Hs00269491_m1	1,2
TNFRSF10B	NM_147187.1	Hs00366272_m1	1,2
TNFRSF10C	NM_003841.2	Hs00182570_m1	1,2
TNFRSF10D	NM_003840.3	Hs00174664_m1	1,2
TNFRSF11A	NM_003839.2	Hs00187189_m1	1,2
TNFRSF11B	NM_002546.2	Hs00171068_m1	1,2
TNFRSF13B	NM_012452.2	Hs00234859_m1	1,2
TNFRSF17	NM_001192.2	Hs00171292_m1	1,2
TNFRSF18	NM_148901.1	Hs00188346_m1	1,2
TNFRSF19	NM_018647.2	Hs00218634_m1	1,2
TNFRSF1B	NM_001066.2	Hs00153550_m1	1,2
TNFRSF25	NM_148965.1	Hs00237054_m1	1,2
TNFRSF4	NM_003327.2	Hs00533968_m1	1,2
TNFRSF7	NM_001242.3	Hs00154297_m1	1,2
TNFRSF8	NM_152942.2	Hs00174277_m1	1,2
TNFRSF9	NM_001561.4	Hs00155512_m1	1,2
CD40	NM_152854	Hs00374176_m1	1
FAF1	NM_131917	Hs00169544_m1	1
FBF1	NM_001080542.1	Hs00384673_m1	1
LTBR	NM_002342	Hs00158922_m1	1
TNFRSF12A	NM_016639	Hs00171993_m1	1
TNFRSF13C	NM_052945	Hs00606874_g1	1
TNFRSF14	NM_003820	Hs00187058_m1	1
TNFRSF19L	NM_032871	Hs00262701_m1	1

Gene	NM number	Assay number	Assay
TNFRSF1A	NM_001065	Hs00533560_m1	1
TNFRSF21	NM_014452	Hs00205419_m1	1
EDA2R	NM_021783.2	Hs00222305_m1	1,2
TRAF1	NM_005658.3	Hs00194638_m1	1,2
TRAF6	NM_145803.1	Hs00377558_m1	1,2
TRAIP	NM_005879.2	Hs00183394_m1	1,2
TANK	NM_133484	Hs00370305_m1	1
TIFA	NM_052864	Hs00385268_m1	1
TRAF2	NM_021138	Hs00184186_m1	1
TRAF3	NM_145726	Hs00237035_m1	1
TRAF3IP2	NM_147200	Hs00210113_m1	1
TRAF4	NM_145751	Hs00188755_m1	1
TRAF5	NM_145759	Hs00182979_m1	1
TRAF7	NM_032271	Hs00260228_m1	1
TTRAP	NM_016614	Hs00213282_m1	1
AVEN	NM_020371.2	Hs00220565_m1	1,2
ENDOG	NM_004435.2	Hs00172770_m1	1,2
ICEBERG	NM_021571.2	Hs00253674_s1	1,2
AIF1	NM_001623	Hs00610419_g1	1
CYC1	NM_001916	Hs00357717_m1	1
DIABLO	NM_138930	Hs00219876_m1	1
HTRA2	NM_013247	Hs00234883_m1	1
DFFB	NM_001004285.1	Hs00237077_m1	1,2
DFFA	NM_213566	Hs00189336_m1	1
DAPK1	NM_004938	Hs00234480_m1	1,2
DAPK1	NM_004938.2	Hs00234489_m1	1,2
DAPK2	NM_014326.3	Hs00204888_m1	1,2
DAPK3	NM_001348.1	Hs00154676_m1	1,2
RALBP1	NM_006788.3	Hs00183639_m1	1,2
RIPK1	NM_003804.3	Hs00169407_m1	1,2
RIPK3	NM_006871.3	Hs00179132_m1	1,2
RIPK4	NM_020639	Hs00221005_m1	1
DAP	NM_004394	Hs00234397_m1	1
DAXX	NM_001141969.1	Hs00154692_m1	1
MIB1	NM_020774	Hs00379185_m1	1
BFAR	NM_016561	Hs00275423_m1	1
CFLAR	NM_003879	Hs00236002_m1	1

Supplementary Table 3a: Differentially expressed genes in the CD34+ cell fraction (patients versus low-risk MDS versus high-risk MDS)

			Ratio of median expression in:		
gene	p-value	effect on apoptosis	low risk MDS/ controls	high risk MDS/ controls	high risk MDS/ low risk MDS
TNFRSF4	0.0023**	anti	0.2*	0.9	4.4**
TNFRSF19	0.0070	pro	0.3	0.1*	0.2
RALBP1	0.031	anti	3.6*	0.9	0.3
TNFRSF7	0.046	pro	0.2*	0.1*	0.4
TRAIP	0.050	pro	1.3	0.4	0.3*
BNIP1	0.050	anti	1.9	0.8	0.4*

Supplementary Table 3b: Differentially expressed genes in the CD71+ cell fraction (patients vs low-risk MDS vs high-risk MDS)

			Ratio of median expression in:		
gene	p-value	effect on apoptosis	low risk MDS/ controls	high risk MDS/ controls	high risk MDS/ low risk MDS
BIK	0.003	pro	215*	203*	0.9
DAPK1	0.006	pro	2.5	6.8*	2.8
DAPK1	0.008	pro	2.4	10.8*	4.5*
BNIPL	0.013	pro	0.1*	0.1*	0.5
TNFRSF10A	0.015	pro	3.2	8.1*	2.5
CARD9	0.017	pro	1.3	3.3*	2.6
BCL2L10	0.043	anti	1.1	3.4*	3.0

Supplementary Table 3c: Differentially expressed genes in the CD13/33+ cell fraction (patients vs low-risk MDS vs high-risk MDS)

			Ratio of median expression in:		
gene	p-value	effect on apoptosis	low risk MDS/ controls	high risk MDS/ controls	high risk MDS/ low risk MDS
TNFRSF13B	0.002	-	0.2*	0.0*	0.0*
MDM2	0.011	anti	1.6*	2.0*	1.2
DAPK2	0.015	pro	0.5	1.9	3.9*
TNFRSF10C	0.029	anti	0.5	1.7	3.5*
CARD6	0.042	anti	0.9	2.3	2.5*

** indicates significant difference (p<0.05) in expression after posthoc analysis*
*** indicates significant difference (p<0.05) using p-value for multiple testing*

Chapter 3

Acquired mutations in *TET2* are common in myelodysplastic syndromes

Nature Genetics 2009; 41 (7): 838-842

SMC Langemeijer*, RP Kuiper*, M Berends, R Knops, MG Aslanyan, M Massop, E Stevens-Linders, P van Hoogen, A Geurts van Kessel, RAP Raymakers, EJ Kamping, GE Verhoef, E Verburgh, A Hagemeijer , P Vandenberghe, T de Witte, BA van der Reijden & JH Jansen

*Contributed equally

Abstract

Myelodysplastic syndromes (MDS) represent a heterogeneous group of neoplastic hematopoietic disorders[1]. Several recurrent chromosomal aberrations have been associated with MDS, but the genes affected have remained largely unknown. To identify relevant genetic lesions involved in the pathogenesis of MDS, we performed SNP-array-based genomic profiling and genomic sequencing in 102 individuals with MDS and identified acquired deletions and missense and nonsense mutations in the *TET2* gene in 26% of these individuals. Using allele-specific assays, we detected *TET2* mutations in most of the bone marrow cells (median 96%). In addition, the mutations were encountered in various lineages of differentiation including CD34+ progenitor cells, suggesting that *TET2* mutations occur early during disease evolution. In healthy tissues, *TET2* expression was shown to be elevated in hematopoietic cells with highest expression in granulocytes, in line with a function in myelopoiesis. We conclude that *TET2* is the most frequently mutated gene in MDS known so far.

Introduction

Myelodysplastic syndromes (MDS) are clonal hematopoietic disorders characterized by dysplasia of the myeloid, megakaryocytic and/or erythroid lineages. The clinical course varies from a smoldering disease to a more acute manifestation with bone marrow failure. In about 30% of cases acute myeloid leukemia (AML) develops, but most affected individuals eventually die from complications of bone marrow failure. Diagnosis of MDS is often difficult as many conditions (such as infection or medication) may give rise to cytopenias and dysplasias without clonal disease. Using karyotyping and fluorescent in situ hybridization (FISH), chromosomal aberrations can be found in approximately 60% of cases[2-4] but information on specific target genes is scarce. Mutations in *NRAS* have been described in 10-15% of the patients, as well as mutations in *TP53* (5-10%), *FLT3* (2-5%), and *RUNX1/AML1* (2-10%)[5]. Recently, haploinsufficiency of *RPS14* has been implicated in impaired erythropoiesis in individuals suffering from MDS with 5q-aberrations[6, 7]. Several SNP array-based genomic profiling studies have been performed yielded various chromosomal regions of interest[8-12]. The aim of this study was to identify novel genes that are recurrently affected in MDS.

Results and discussion

DNA from bone marrow cells of 102 MDS patients was hybridized to Affymetrix 250k SNP-arrays. Genomic profiles were analyzed for copy number abnormalities and copy-neutral loss of heterozygosity (LOH), also referred to as uniparental disomy (UPD). Regions showing acquired UPD usually extend to one of the telomeres and are believed to harbor homozygously mutated genes. In line with known cytogenetic data, we frequently observed aberrations affecting chromosomes 5, 7, 8 and 20 (**Figure 1, Supplementary Table 1**). In addition, microdeletions on chromosome 21 involving *RUNX1* were present in three subjects (**Supplementary Figure 1**). Segmental telomeric UPD was seen on chromosomes 1, 4, 6, 7 and 17 (**Figure 1 and Supplementary Table 2**); a homozygous mutation of *TP53* was detected within the homozygous region in the individual with UPD17p (**Supplementary Figure 2**). UPD was particularly frequent on chromosome 4q. Notably, chromosomal aberrations involving 4q have been described[9-13], but no specific target gene in this region has yet been implicated in MDS. Therefore, we set out to identify the target gene(s) on chromosome 4.

Five individuals with MDS carried large aberrations on the long arm of chromosome 4, of which four (UPN 52, 73, 74 and 100) showed UPD of the 4q arm and one (UPN 7) showed a mono-allelic deletion (**Figure 2a**). None of these abnormalities was observed in a cohort of 1015 healthy controls that we use as an in-house control panel for SNP-array studies. In one patient (UPN 44) a microdeletion (<850 kb) was present at 4q24, which was validated by FISH (**Figure 2b**). Two genes are located within this microdeletion, the pyrophosphatase gene *PPA2* and the ten-eleven-translocation gene *TET2* (*KIAA1546*, *FLJ20032)*. Genomic sequencing did not reveal any mutation in *PPA2*, but the remaining copy of *TET2* harbored a nonsense mutation in most cells, introducing

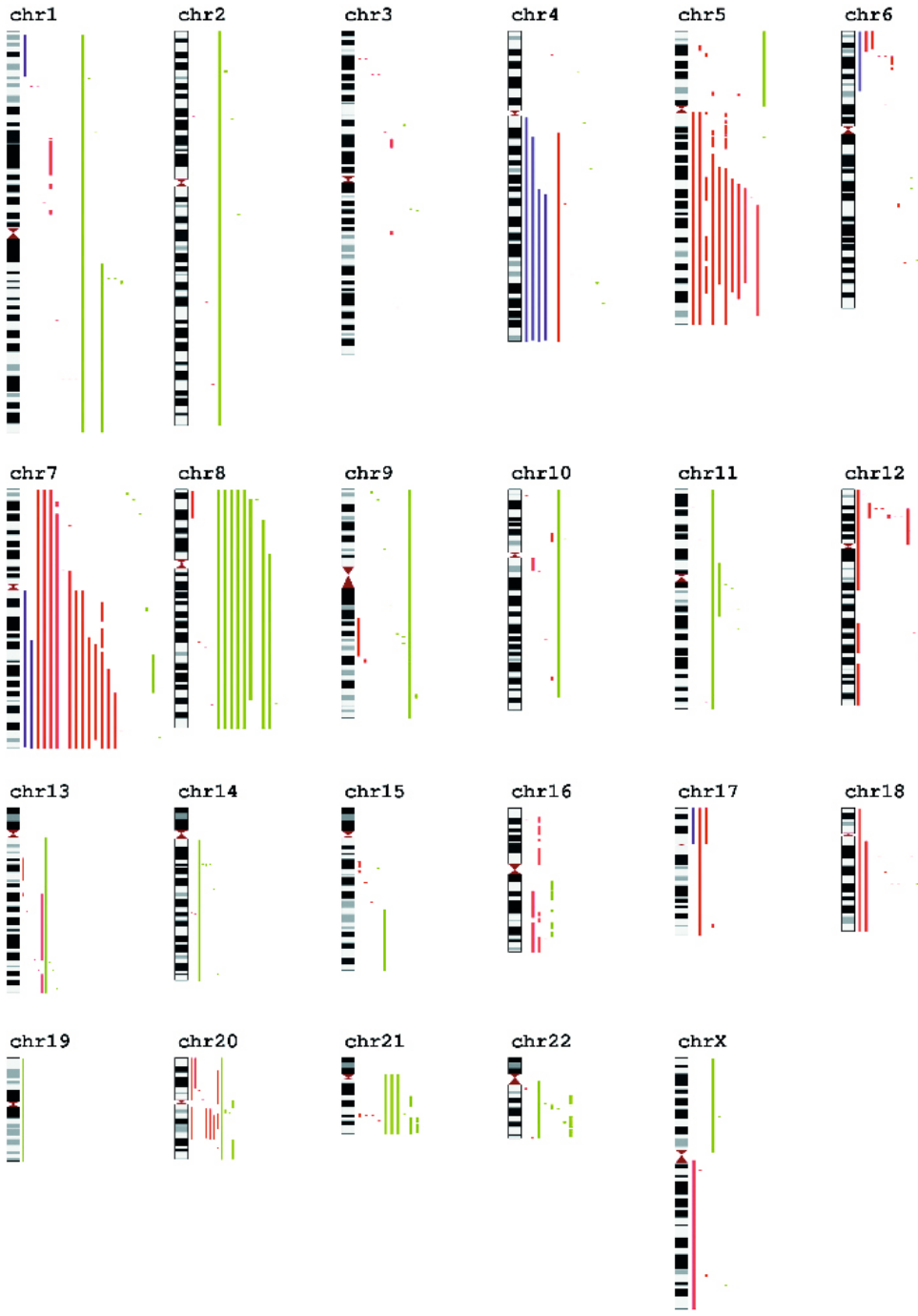


Figure 1: Copy number changes and uniparental disomy in MDS patients.
Overview of all genetic aberrations found with 250k SNP array analysis in 102 MDS cases. Each line represents a different subject. Blue lines are large (> 20Mb) telomeric regions of (acquired) uniparental disomy, red lines represent deletions, and green lines are copy-number gains.

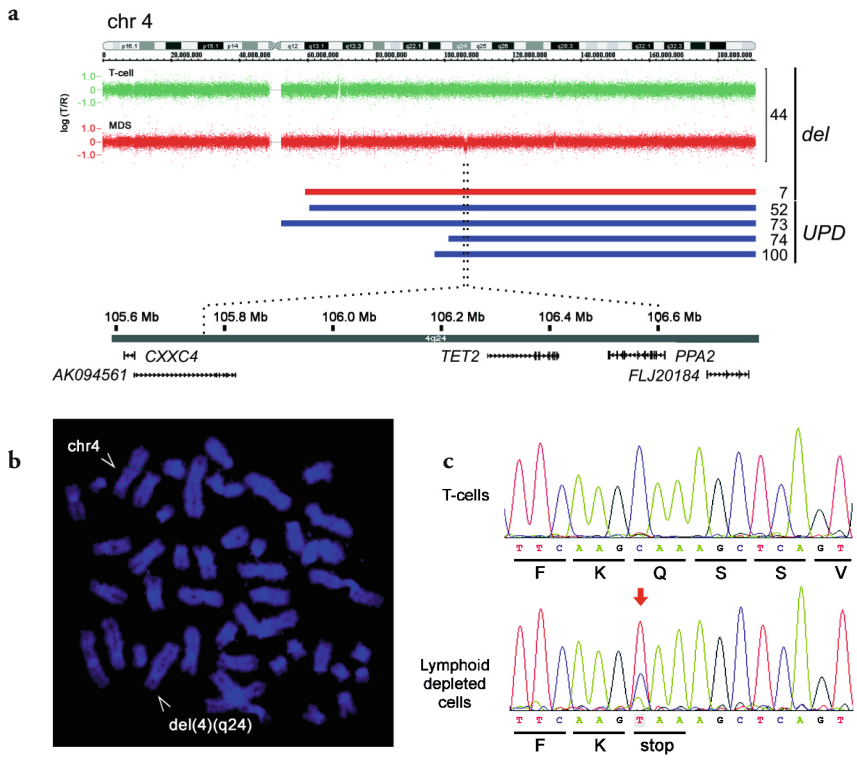


Figure 2: TET2 is recurrently affected in MDS.
(a) Genomic profile of chromosome 4 in patient 44 showing a 850-kb microdeletion on 4q24, encompassing the PPA2 and TET2 genes. This deletion was absent in the T cells of the same individual. Five other subjects carried larger aberrations on 4q, including a deletion (red bar) or uniparental disomy (UPD; blue bars)
(b) Validation of the 4q24 microdeletion in subject 44 by fluorescent in situ hybridization, showing presence of the 4q24 locus (green signal) on only one of the two chromosomes 4 (red centromere probe) in a metaphase of bone marrow cells.
(c) Sequence analysis of DNA from the lymphocyte-depleted cell fraction of patient 44 reveals a C > T conversion at position 1147 of the TET2 gene in the remaining 4q24 allele leading to a premature stop codon (TAA). The mutation was found to be present in a predominant subpopulation of the cells and was not detectable in the non-neoplastic T cells of the same individual.

a premature stop codon (**Figure 2c and Table 1**). Both the 4q24 microdeletion and the nonsense mutation were absent in non-neoplastic T-lymphocytes of the same individual (**Figures 2a and 2c**), indicating that both anomalies were acquired. To establish whether the other five subjects with 4q24 abnormalities also carried *TET2* mutations, we sequenced this gene and found that all five carried bi-allelic anomalies of *TET2* (**Table 1 and Figure 3**). We conclude that *TET2* is the target gene in MDS-affected individuals with 4q24 abnormalities.

To reveal the overall frequency of *TET2* mutations in MDS, we subsequently sequenced the gene in all cases from our cohort. Common single nucleotide polymorphisms (SNPs) present in public databases and/or detected in a cohort of 104 healthy Dutch controls were excluded (**Supplementary Table 3**). In total, we found *TET2* aberrations in 27 out of 102 subjects with MDS (26%, **Table 1**). To investigate whether the mutations were acquired, we analyzed germ-line DNA from T-cells wherever

Table 1: Characteristics of TET2 mutations.

UPN	Classification			Karyotype	SNP array		Mutation analysis by genomic sequencing				
	FAB	WHO	IPSS		UPD at 4q24	del at 4q24	Nonsense	Missense	Indel	Homo/Hetero/ hemizygous	Acquired/ inherited
44	RAEB	RAEB-2	int-2	normal	no	4q24.23	Q383X			hemizygous	acquired
46	RA	RCMD	low	normal	no	no	E961X	R1896S	F1285del	heterozygous heterozygous heterozygous	acquired
50	RA	RCMD	low	normal	no	no	Q417X			heterozygous	acquired
52	RAEB	RAEB-1	int-1	del(9) (q22q32)	4q	no	S842X			homozygous	acquired
59	RAEB	RAEB-1	int-2	-7	no	no	R506X			heterozygous	acquired
72	RAEB	RAEB-1	int-1	normal	no	no	R544X	C1875R		heterozygous heterozygous	acquired
73	RAEB	RAEB-1	int-1	+8	4q12-q35.2	no		W1291R		homozygous	acquired
75	RAEB	RAEB-1	int-1	normal	no	no	E368X S1848X			heterozygous heterozygous	acquired
77	RAEB	RAEB-1	int-1	normal	no	no		L1398R	L1240fsX2	heterozygous heterozygous	acquired
79	RARS	RCMD-RS	int-1	normal	no	no		G1913D		heterozygous	acquired
81	RA	RCMD	int-1	normal	no	no			Q574fsX5	heterozygous	N/A
83	RA	MDS-U	low	normal	no	no	C1271X		T1220fsX5	heterozygous heterozygous	N/A
86	RA	RA	low	normal	no	no			N608fsX30 K1493fsX77	heterozygous heterozygous	N/A
87	RA	RA	low	normal	no	no			Q847fsX24	heterozygous	N/A
96	RA	RCMD	low	normal	no	no	R1216X	R1214W†		heterozygous heterozygous	N/A
97	RA	RCMD	int-1	normal	no	no		C1396W† I1873T†		heterozygous heterozygous	N/A
98	RA	RA	low	normal	no	no			P555fsX10	heterozygous	N/A
100	RA	RCMD	low	normal	4q22.3-q35.2	no			H682fsX10	homozygous	N/A
101	RA	RA	low	normal	no	no			K1911_ L1916del	heterozygous	N/A
102	RA	RCMD	low	normal	no	no		R1261L†		heterozygous	N/A
103	RAEB	RAEB-1	int-1	normal	no	no			L1819fsX T164fsX5	heterozygous heterozygous	N/A
104	RAEB-t	AML	high	Complex***	No	no			S826fsX14	heterozygous	N/A
7	RAEB-t	RAEB-2	int-2	complex	no	4q23-q35.2		P1962L†		heterozygous**	N/A
9	RA	RCMD	low	normal	no	no			C1271fsX28 S1424fsX23	heterozygous heterozygous	N/A
11	RARS	RCMD-RS	int-1	+8	no	no		H1881Q†		heterozygous	N/A
36	RARS	RCMD-RS	low	normal	no	no			T996fsX12	heterozygous	N/A
74	RAEB-t	AML MLD	high	normal	4q23-q35.2	no			Q1170fsX55	homozygous	N/A

* *In 27 of 102 individuals with MDS, aberrations of TET2 were observed in bone marrow cells. Uniparental disomy (UPD), amplifications and deletions at 4q24 were analyzed using SNP-arrays. Genomic sequencing of the protein coding region and splice donor and acceptor sites revealed nonsense, missense and insertion/deletion mutations leading to an amino acid deletion and substitution or to a frameshift resulting in a stopcodon after 2-77 aminoacids (indicated as fsX followed by the number of aminoacids18). Sequence aberrations were classified as heterozygous, homozygous or hemizygous by analyzing the intensity of the base pair signal in the sequence reaction. Whenever possible, purified T cells were analyzed to assess whether the observed mutations were acquired. UPN, unique patient number. N/A, not analyzed due to lack of sufficiently available T cells. Nucleotide positions of the mutations are indicated in Supplementary Table 5.*

** *Sequencing showed both the missense as well as the wild type sequence (albeit with lower signal intensity). As the SNP array showed loss of one allele, the wild type sequence might have arisen from remaining non-clonal bone marrow cells.*

*** *Including deletion of chromosome 4 in 2/39 cells analyzed by conventional karyotyping.*

† *Although these missense mutations were not present in our cohort of healthy controls and did result in changes of conserved amino acids (Supplementary Figures 3 and 4), it cannot be excluded that they represent rare SNPs, since germ-line material of these patients was not available.*

available (10/27). In all cases examined, the mutations were restricted to the nonlymphoid cells, indicating that they were acquired. Nonsense and frameshift mutations leading to premature stop codons were scattered throughout the *TET2* coding sequence, with preference for the N-terminal and middle moieties of the protein (Fig. 3). Notably, all missense mutations leading to amino acid substitutions and in-frame deletions clustered in two distinct regions (**Figure 3**). Alignment of *TET2* orthologues from different species showed that these regions are highly conserved (**Supplementary Figure 3**). In addition, these regions were found to be conserved in the human *TET2* paralogues *TET1* and *TET3* (**Supplementary Figure 4**), indicating that they represent functionally important domains.

Notably, it was very recently found that *TET2* mutations in myeloproliferative neoplasms target both multipotent and committed progenitor cells, suggesting that *TET2* has an important regulatory role for *TET2* in myelopoiesis[14]. To gain further insight into its function, we studied *TET2* expression in various cell types. First, we determined whether the different splice variants that are predicted by genome databases are expressed. Three isoforms were shown to be expressed, of which isoforms 2 and 3 lack the C-terminal part of the protein, including the two highly conserved regions (**Figure 4a and Supplementary Figure 5**). *TET2* showed a broad expression pattern in different tissues, with a 10-100 fold higher expression in hematological cells, particularly in granulocytes (**Figure 4b**). In addition, *TET2* expression was upregulated during all-*trans* retinoic acid induced granulocytic differentiation of the promyelocytic cell line NB4 (**Figure 4c**), underscoring its proposed role in myeloid differentiation. To compare the expression of *TET2* in MDS cases and healthy controls, we isolated CD34+ cells, CD13+/33+ myelo-monocytic cells and CD71+ erythroid cells from bone marrow, and mature granulocytes from peripheral blood. *TET2* expression was comparable between most fractions from MDS cases and controls, but was significantly decreased in the granulocytes from MDS cases (**Figure 4d**, $p<0.01$). This decreased *TET2* expression was observed in all MDS-affected individuals, irrespective of the *TET2* mutation status. As all subjects with MDS showed morphological defects of the mature granulocytes (**Supplementary Table 4**), the decreased *TET2* expression levels may be caused by a disturbed or incomplete granulocytic differentiation in the MDS cells, thereby further linking *TET2* expression to myeloid differentiation.

Subject 44 later progressed to AML. Notably, in the leukemic blasts, the 4q24 deletion and the nonsense *TET2* mutation were retained, but an additional 7q deletion (associated with poor

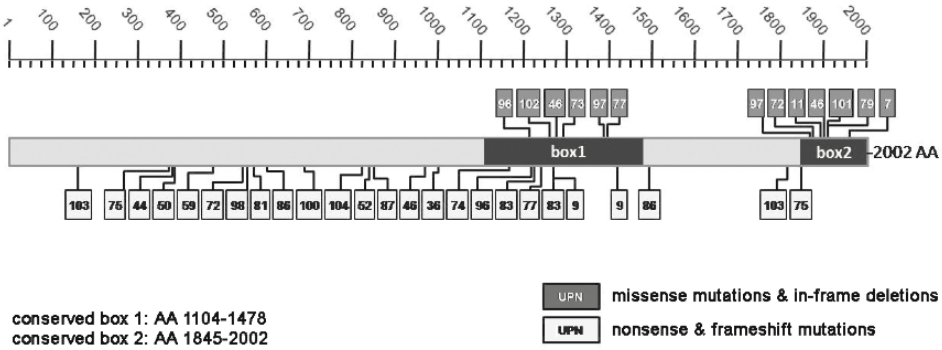


Figure 3: Localization of TET2 mutations.
Localization of all TET2 mutations detected in 27 individuals with MDS shown at the protein level. Missense mutations are indicated by dark gray squares, nonsense and frameshift mutations in light gray squares. All missense mutations are located in one of the conserved protein regions. These conserved regions are present in isoform 1, but absent in isoforms 2 and 3. UPN, unique patient number (corresponding to Table 1); AA, amino acid.

prognosis in MDS) plus a gain on chromosome 11q were acquired at the time of AML progression (**Supplementary Figure 6**). This indicates that the bi-allelic *TET2* inactivation represented an earlier event than the aberrations on 7q and 11q. To further address whether *TET2* mutations represent early or late events in the course of the disease, we conducted allele-specific, quantitative assays for the different mutations in 25 of 27 cases (**Figure 5a, Supplementary Tables 5a and 5b and Supplementary Figures 7 and 8**). Although in five cases the allelic burden was between 56 and 70%, in most patients with mono-allelic mutations, the mutant allele was present in the majority (>70%) of the cells, suggesting that the mutation was an early event (overall range: 56-100%, median 96%). In cases with more than one *TET2* mutation, at least one mutant allele was present in most of the cells whereas the second mutation was present in variable percentages (range 22-100%, median 80%) showing that acquisition of a second mutation may lead to further expansion of double-positive subclones. In one subject (UPN46), three different *TET2* mutations were detected in 96%, 43% and 44% of the cells respectively, suggesting that two different subclones, each with a different second mutation, had arisen from the initial mutant clone.

Next, we determined in which bone marrow cells the *TET2* mutants were expressed. To this end, we isolated RNA from CD71+ erythroid precursors, CD13/33+ myelomonocytic cells and CD34+ progenitor cells from the bone marrow of seven MDS-affected individuals with a *TET2* mutation. In all cases, mutant *TET2* was found to be present in all subfractions tested. Of note, we detected a second mutation in patient UPN 77 in the CD34+ and CD13/33+ cells, but not in the CD71+ cells (which represented <10% of the total bone marrow cells)(**Supplementary Figure 9 and Supplementary Table 6a and 6b**). This observation further indicates that *TET2* mutations represent early events, and that second mutations in *TET2* may occur in subclones arising at later stages of disease development.

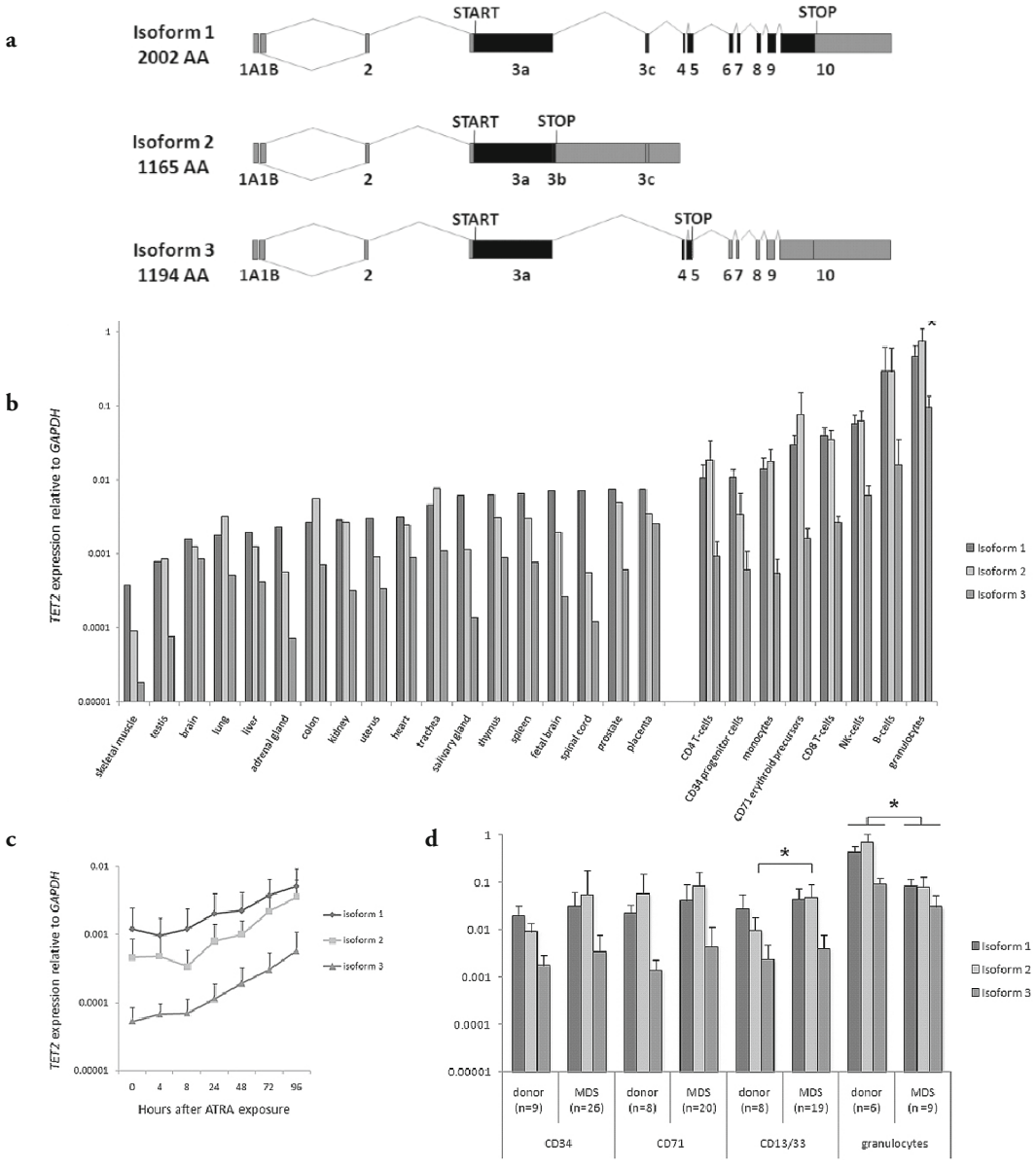


Figure 4: Expression of three TET2 isoforms in various cells and tissues.

(a) Schematic representation of the exon structure of three isoforms of TET2 as confirmed by RT-PCR. Location of the (shared) start codons and the stop codons are indicated. The translated region is indicated in black. AA, amino acid.

Expression of TET2 isoforms was measured by isoform-specific quantitative PCR. Expression is depicted relative to the housekeeping gene GAPDH. The mean expression of TET2 isoforms is indicated. (b) Expression was determined in various tissues (pooled RNA) and hematopoietic cell fractions. The expression is significantly higher in granulocytes than in other hematopoietic cells (indicated by an asterisk).(c) The promyelocytic cell line NB4 before and after induction of granulocytic differentiation using all-trans retinoic acid (ATRA, 10^{-6} M). The difference in time is statistically significant ($p<0.05$). d) Expression in various hematopoietic subfractions of MDS cases and controls. In the CD13/33 fraction, expression of isoform 2 was significantly different in cases compared to controls. In the granulocyte fraction, expression of all three isoforms was significantly decreased. An asterisk (*) indicates statistically different expression ($p<0.05$). Error bars depict standard deviation.

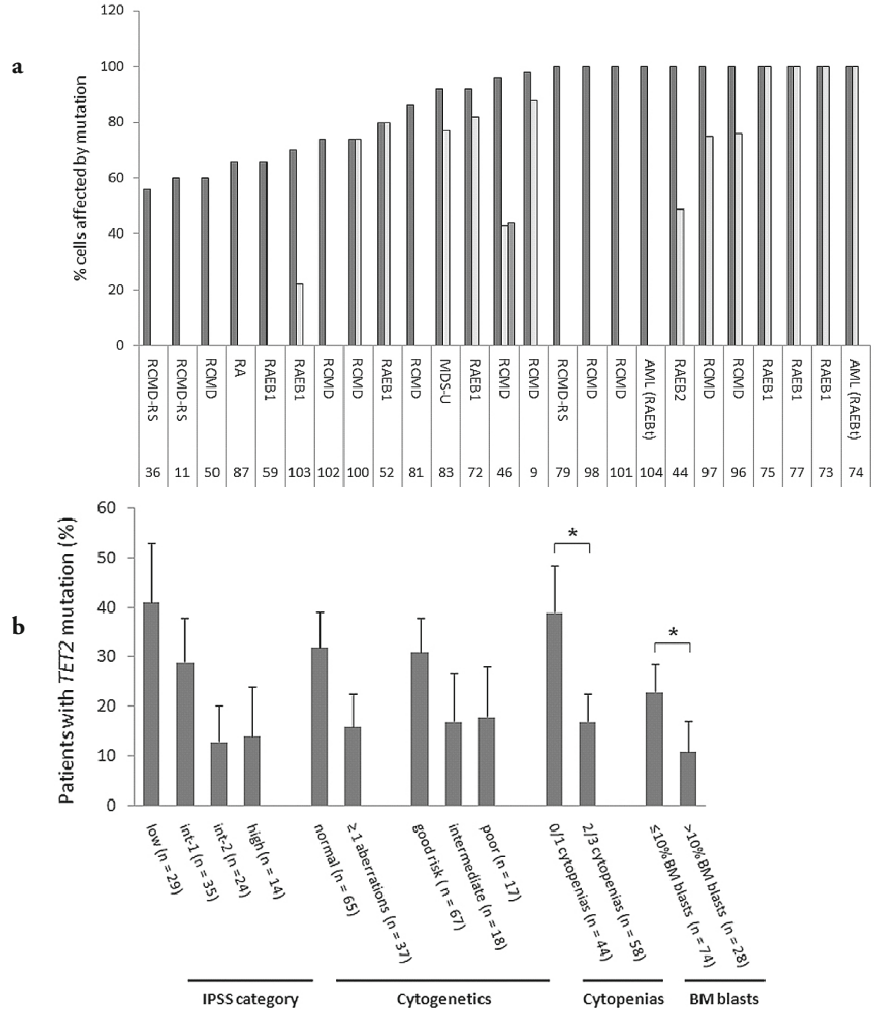


Figure 5: Correlation of TET2 mutation status and allelic burden with clinical phenotype.

(a) Allelic burden of the identified TET2 mutations (and deletion in case of UPN 44). In case of multiple mutations in a single individual, the allelic burden of all mutations is shown by different bars. Data are sorted based on an increasing allelic burden.

*(b) The incidence of TET2 mutations is shown in the four IPSS subgroups. As the IPSS is determined by the cytogenetic findings, cytopenias and amount of bone marrow blasts, these subgroups are shown separately. Also, patients with a normal karyotype and with one or more aberrations are distinguished. As shown, the frequency of TET2 mutations varies between the subgroups. *P < 0.05 was considered statistically significant. Error bars, s.d.*

TET2 mutations occurred in all subtypes defined by the International Prognostic Scoring System (IPSS), but were more frequent in the low (41%) and int-1 risk categories (27%) as compared to the int-2 (13%) and high risk (14%) groups (**Figure 5b**). *TET2* mutations were significantly less common in patients with multiple cytopenias or high blast cell counts (**Figure 5b**). Monoallelic or biallelic mutation status and the mutant allelic burden did not significantly correlate with MDS subcategory (**Figures 5a and Supplementary Table 5**), overall survival or progression to AML in this cohort (data not shown), but to properly address this point, a larger prospective study is needed.

To assess whether *TET2* is affected in a wider range of myeloid malignancies, we sequenced *TET2* in a cohort of individuals with *de novo* AML (n=32) (**Supplementary Table 7**). *TET2* mutations were indeed detected in 6 of 32 patients and co-occurred with various other cytogenetic and molecular aberrations, including *PML-RAR* translocations and *NPM1* mutations. Together with the above mentioned observation that *TET2* mutations occur in myeloproliferative disease as well, our data suggest a general role for this gene in myeloid malignancies.

In conclusion, we have identified *TET2* as the most frequently mutated gene in MDS thus far known. As in all cases at least one, and in many cases two alleles were affected, loss of function seems to be the most probable mechanism by which these mutations contribute to the malignant transformation of bone marrow cells, thereby defining *TET2* as a previously undescribed tumor-suppressor gene in MDS.

Methods

Patients and healthy controls

Bone marrow (n=102) and blood (n=10) from MDS patients and blood from healthy controls (n=104) were collected after informed consent. Consecutive patients from the Radboud University Nijmegen Medical Center Nijmegen (n =83) belonging to all different WHO and IPSS categories and cytogenetic subgroups were included. A second cohort of karyotypically normal patients from the University Hospital Leuven (n=19) was also included. Patient characteristics are shown in Supplementary Table 8. Cells were frozen in liquid nitrogen until further use. DNA was extracted from FACS-sorted lymphocyte-depleted bone marrow (patients, n=41), Ficoll density gradient isolated mononuclear cells from bone marrow (patients, n= 61), or white blood cells after NH_4Cl lysis (controls, n=104). In addition, in a subset of patients (n=10) DNA was extracted from T-cells isolated from peripheral blood by FACS sorting of CD3+/CD19-/CD45+ cells. In case only limited amounts of cells were available, in vitro expansion of polyclonal T-cells was performed. For this purpose, cells were cultured in IMDM and 10% Human Serum for 7-14 days in medium containing IL-2 (100 IU/ml) and beads coated with anti-CD3 and anti-CD28 (Dynabeads, Invitrogen). After culture, the purity of the T-cells was measured using CD3 and CD45 surface markers. To ensure high purity, sorting of the CD3+ cells by FACS was performed. DNA was isolated using Qiagen spin-columns and its quality was checked on gel and by using a nanodrop ND-1000 spectrophotometer analysis.

Granulocytes were isolated after 1077 Ficoll density gradient centrifugation and erythrocyte lysis. The other cell subtypes were isolated by FACS sorting using monoclonal antibodies directed against CD4 and CD8 (T-cells), CD14 (monocytes), CD19 (B-cells), CD71 (erythroid cells), CD34 (progenitor cells), CD56 (NK-cells), CD13/33 (mono/myeloid-cells). Pooled RNA from all other tissues was commercially obtained (Clontech). NB4 cells were cultured in RPMI medium supplemented with fetal bovine serum and penicilline/streptomycine and exposed to 10^{-6} M *all-trans* retinoic acid (ATRA)(n = 3).

Bone marrow (n=21) and blood (n=11) of AML patients were collected after informed consent. Patients were diagnosed in the Radboud University Nijmegen Medical Center (n=25) or the Leiden

University Medical Centre (n=7). We included patients of all FAB subtypes. Since AML M3 represents an important subgroup of AML, characterized by the PML-RAR translocation and its unique sensitivity to ATRA, a relatively large cohort of M3 patients was examined.

SNP-array analysis

Affymetrix 250k SNP arrays were hybridized according to the protocol provided by the manufacturer (Gene Chip Mapping 500K Assay manual). Briefly, 250 µg of total genomic DNA was digested using the Nsp I restriction enzyme and ligated to adaptors. A single primer that recognizes the adaptor sequence was used to amplify adaptor ligated DNA fragments. The amplified DNA was purified, digested with DNase I and labeled with biotin. Subsequently, samples were hybridized to GeneChip Human mapping 250k arrays (Affymetrix). The arrays were washed and stained by streptavidin-phycoerythrin conjugates using the Affymetrix GeneChip Fluidics Station 450. Subsequently, the arrays were scanned with the Affymetrix GeneChip Scanner 3000 to obtain the fluorescence intensity data. Analysis of data, including quality control, was performed with the Genotyping Analysis Software GTYPE (Affymetrix). Copy number analysis was performed using the public domain CNAG software package[15]. Next to visual inspection, the Hidden Markov Model (HMM) available within the CNAG software package was used to score the copy number aberrations. An overview plot was made using the Genome-Wide Viewer available at https://bioinformatics.cancerresearchuk.org/ ~cazier01/GWA_View.html. For the analysis of DNA from the T-cell fraction, the mononuclear MDS fraction, and an AML diagnosis sample from patient UPN44, the Affymetrix SNP6.0 arrays, containing 1.8 million SNPs and copy number probes, were used. Genotypes were generated using Birdseed analysis software implemented in the Affymetrix Genotyping Consule v2.1. To exclude those regions that show normal copy number variation all detected copy number aberrations were compared to those present in paired T-cells (n=8), our in-house panel of copy-number variations in healthy controls (currently containing 1015 individuals), as well as those previously reported in 270 unaffected HapMap individuals[16] and the Database of Genomic Variants (http://projects.tcag.ca/variation). Copy number neutral loss of heterozygosity (LOH) was defined as areas consisting of stretches of >70 sequentially homozygous SNPs. The regions thus defined were ordered based on size and location (i.e. interstitial or telomeric).

FISH analysis

DNA from BAC clones RP11-449G2 and RP11-542F11 (BACPAC Resources, Oakland, USA), both spanning the *TET2* gene, were labelled with biotin and immunocytochemically stained with FITC. As a reference, a Cy3-labeled chromosome 4 centromeric probe was used (Vysis Inc.). Visual examination of the slides was performed using a Zeiss Axiophote-2 microscope equipped with appropriate filters. Twenty metaphases were examined. Digital images were captured using a high-performance Leica DC 350FX camera coupled to Leica CW 4000 software package.

Sequence analysis

Sequence analysis was performed on PCR-amplified genomic DNA fragments spanning the entire coding region of all three *TET2* isoforms (for primer sequences, see **Supplementary Table 9**). All intron-exon boundaries were included to identify possible splice site aberrations. All products were sequenced bi-directionally and sequence variations were confirmed by an independent PCR and sequencing procedure starting from the original patient DNA. To analyze whether the observed mutations were acquired, DNA from T-cells, if available, was isolated from patients showing sequence variations in *TET2* (n=10). To further minimize the chance that putative mutations represent SNPs, the observed mutations were compared with known ESTs. Moreover, *TET2* was sequenced in a group of 104 healthy Dutch controls.

Determination of allele burden

To establish the allele burden of *TET2* mutations, pyrosequencing (PSQ) reactions were performed in patients with missense mutations and single basepair deletions/insertions according to the protocol of the manufacturer, with minor modifications[17]. Briefly, ratios of single-nucleotide missense and nonsense mutations relative to the wild-type sequence were determined using a Pyrosequencer PSQ96MA platform (Biotage AB, Uppsala, Sweden). Mutation-specific PSQ primer combinations were developed in-house, using dedicated 'PSQ Assay Design' software (Isogen Life Science). Mutation-specific PSQ primer sequences are shown in Supplementary Table 9. All PSQ reactions were performed in duplicate.

Genescan analysis was performed in MDS patients with *TET2* microdeletions consisting of two or more nucleotides found by sequencing (n=4) and in patients with single basepair deletions/insertions where pyrosequencing was unsuccessful (n =3). The primers used for genescan analysis are shown in Supplementary Table 9. Briefly, 50 PCR cycles were performed using unlabeled primers, followed by 15 cycles with a labeled forward or reverse primer (conditions: 5 min. at 95°C, 50/15 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, followed by 10 min at 72°C). 1 µl of each PCR product was analyzed using the 3730 DNA analyzer (Applied Biosystems) and Peak Scanner Software (Applied Biosystems).

Splice-variant analysis

To confirm the expression of the *TET2* splice-variants described in databases (*TET2* Isoform 1: NM_001127208 / UniRef100Q6N021. Isoform 2: NM_017628 / UniRef100Q6N021-2. Isoform 3: UniRef100_Q6N021-3), primer pairs were designed covering all relevant, isoform specific, exon-exon boundaries (for primer sequences, see **Supplementary Table 9**). PCR was performed on cDNA followed by sequencing. All products were sequenced in two directions.

Quantitative PCR

To assess the mRNA expression of *TET2* in different hematopoietic cell fractions and tissues, specific PCR primers and probes were designed that discriminate between the three different isoforms of *TET2* (**Supplementary Table 9**). Quantitative PCR was performed using an Applied Biosystems Taqman 7900HT machine. Quantities were normalized using GAPDH expression as a reference and calculated using the $2^{\Delta CT}$ method.

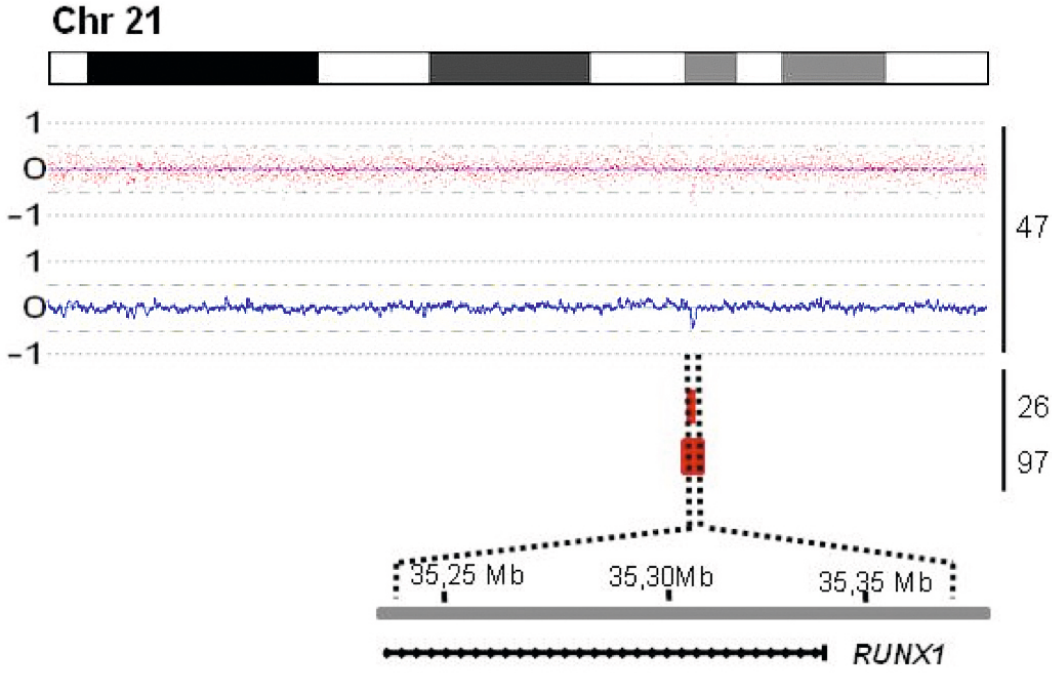
Statistical analysis

The occurrence of *TET2* mutations in different patient subgroups was determined. Error bars show the standard deviation. P-values were calculated using a Pearson chi square test (4x2 and 3x2 tables) or a two-tailed Fisher exact test. Statistical analysis of *TET2* expression in NB4 cells during differentiation was performed with ANOVA using repeated measures. Statistical analysis of *TET2* expression in various cellular subsets was performed with ANOVA using univariate analysis. P-values <0.05 were considered statistically significant.

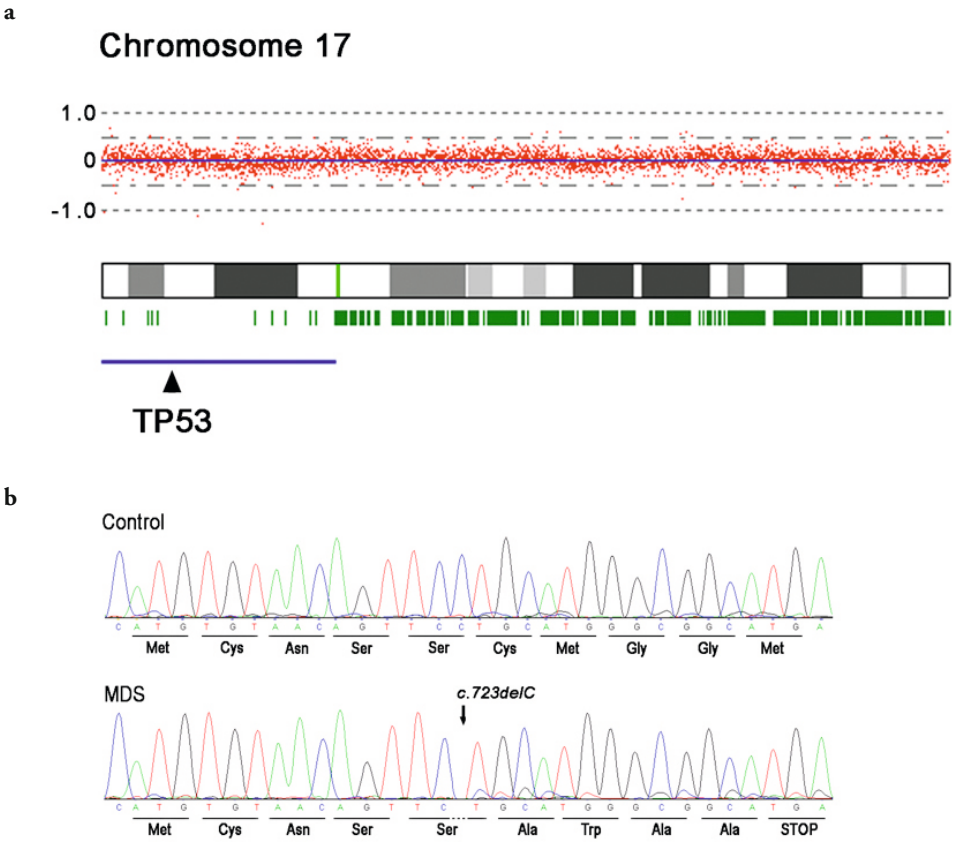
Reference List

1. Nimer,S.D. Myelodysplastic syndromes. Blood 111, 4841-4851 (2008).
2. Pedersen-Bjergaard,J., Pedersen,M., Roulston,D., & Philip,P. Different genetic pathways in leukemogenesis for patients presenting with therapy-related myelodysplasia and therapy-related acute myeloid leukemia. Blood 86, 3542-3552 (1995).
3. List,A.F., Vardiman,J., Issa,J.P., & DeWitte,T.M. Myelodysplastic syndromes. Hematology. Am. Soc. Hematol. Educ. Program.297-317 (2004).
4. Haase,D. et al. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. Blood 110, 4385-4395 (2007).
5. Nolte,F. & Hofmann,W.K. Myelodysplastic syndromes: molecular pathogenesis and genomic changes. Ann. Hematol. 87, 777-795 (2008).
6. Ebert,B.L. et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. Nature 451, 335-339 (2008).
7. Pellagatti,A. et al. Haploinsufficiency of RPS14 in 5q- syndrome is associated with deregulation of ribosomal- and translation-related genes. Br. J. Haematol. 142, 57-64 (2008).
8. Gondek,L.P. et al. Single nucleotide polymorphism arrays complement metaphase cytogenetics in detection of new chromosomal lesions in MDS. Leukemia(2007).
9. Gondek,L.P. et al. Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML. Blood 111, 1534-1542 (2008).
10. Mohamedali,A. et al. Prevalence and prognostic significance of allelic imbalance by single-nucleotide polymorphism analysis in low-risk myelodysplastic syndromes. Blood 110, 3365-3373 (2007).
11. Wang,L. et al. Genome-wide analysis of copy number changes and loss of heterozygosity in myelodysplastic syndrome with del(5q) using high-density single nucleotide polymorphism arrays. Haematologica 93, 994-1000 (2008).
12. Dunbar,A.J. et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res. 68, 10349-10357 (2008).
13. Viguie,F. et al. Common 4q24 deletion in four cases of hematopoietic malignancy: early stem cell involvement? Leukemia 19, 1411-1415 (2005).
14. Delhommeau,F. et al. Mutation in TET2 in myeloid cancers. N. Engl. J. Med. 360, 2289-2301 (2009).
15. Nannya,Y. et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res. 65, 6071-6079 (2005).
16. Redon,R. et al. Global variation in copy number in the human genome. Nature 444, 444-454 (2006).
17. Ruiter,E.M. et al. The mitochondrial 13513G > A mutation is most frequent in Leigh syndrome combined with reduced complex I activity, optic atrophy and/or Wolff-Parkinson-White. Eur. J. Hum. Genet. 15, 155-161 (2007).
18. den Dunnen,J.T. & Antonarakis,S.E. Nomenclature for the description of human sequence variations. Hum. Genet. 109, 121-124 (2001).

Supplementary information

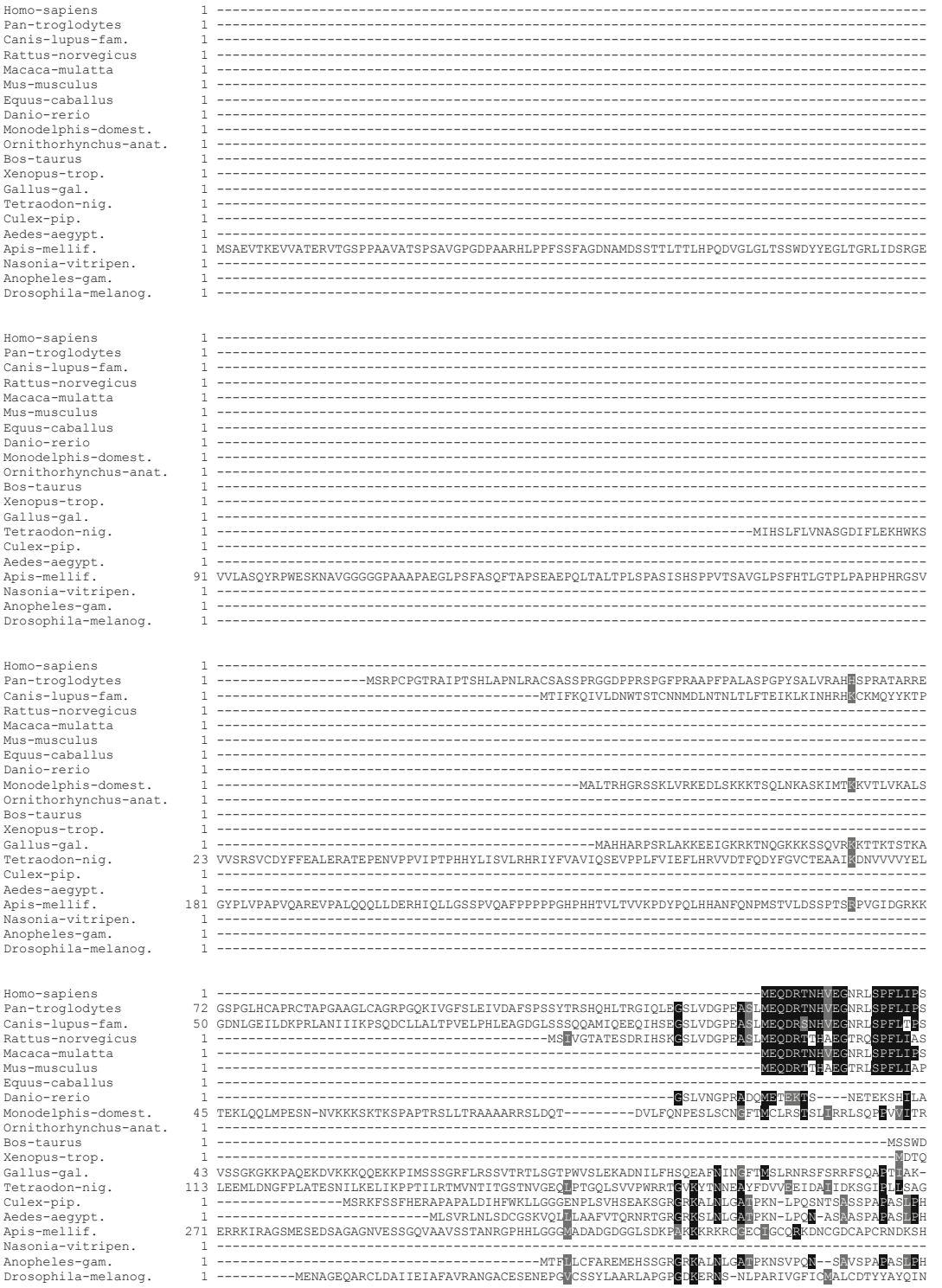


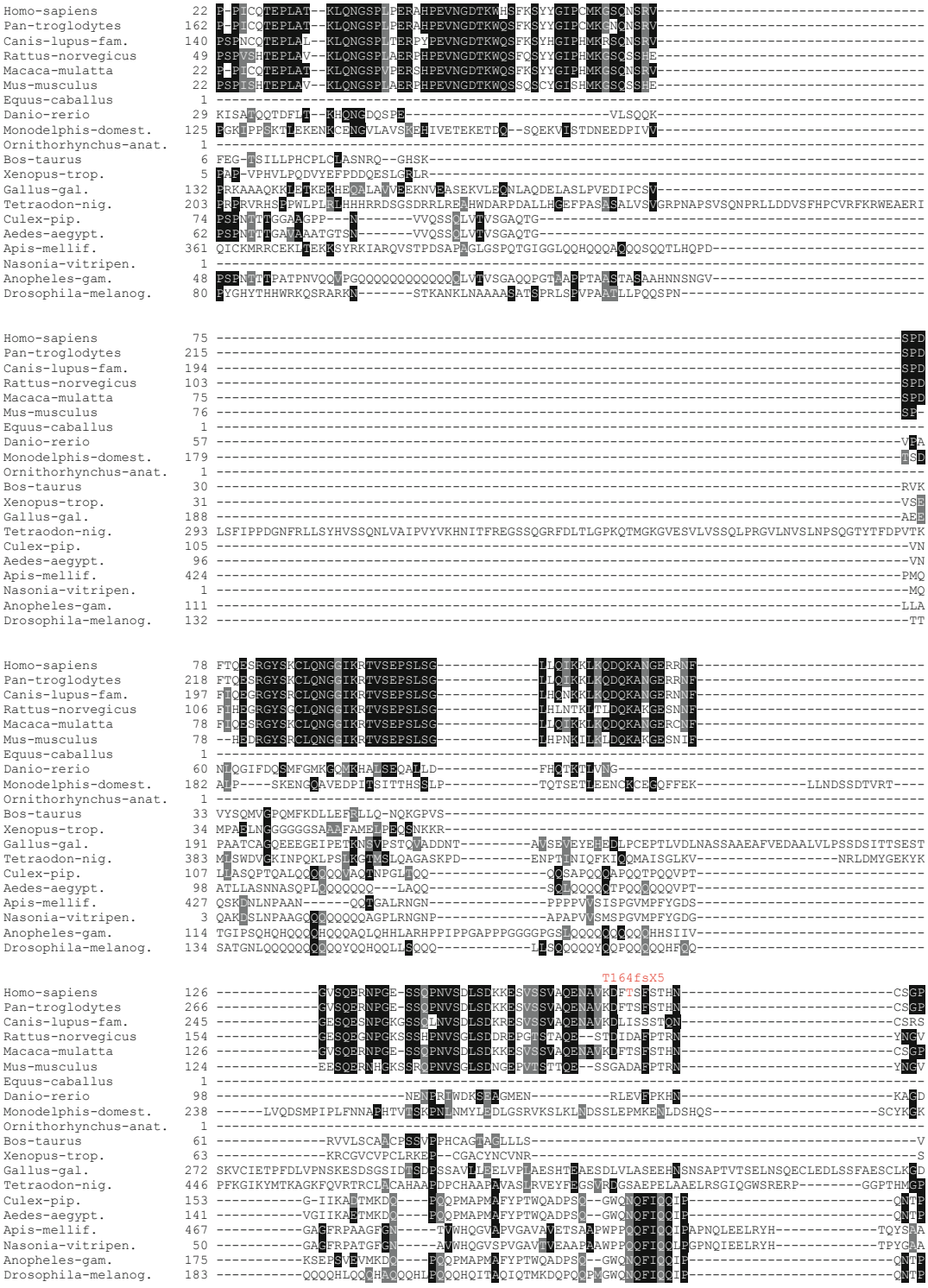
Supplementary Figure 1: RUNX1 deletions detected by 250k SNP-array analysis
In three MDS patients (UPN 47, 26, 97) a microdeletion was detected on chromosome 21 using 250k SNP-array based analysis. The minimal overlapping region contained (part of) RUNX1 as the only affected gene, as indicated below. RUNX1 is known to be affected by mutations in 2-10% of MDS patients. The upper graph shows the genomic profile on chromosome 21 of patient 47. The red boxes indicate the location of the deletions in patients 26 and 97. In patient 97, 9 genes were deleted including RUNX1. In the other two patients only part of RUNX1 was deleted. The exact locations of the deletions is listed in Supplementary Table 1. Although cryptic translocations cannot be excluded, no evidence was obtained for translocations involving RUNX1 upon karyotype analysis (n=3). RUNX1-RUNX1T1 PCR was performed on RNA of patient 47. There was no evidence for a RUNX1-associated translocation.

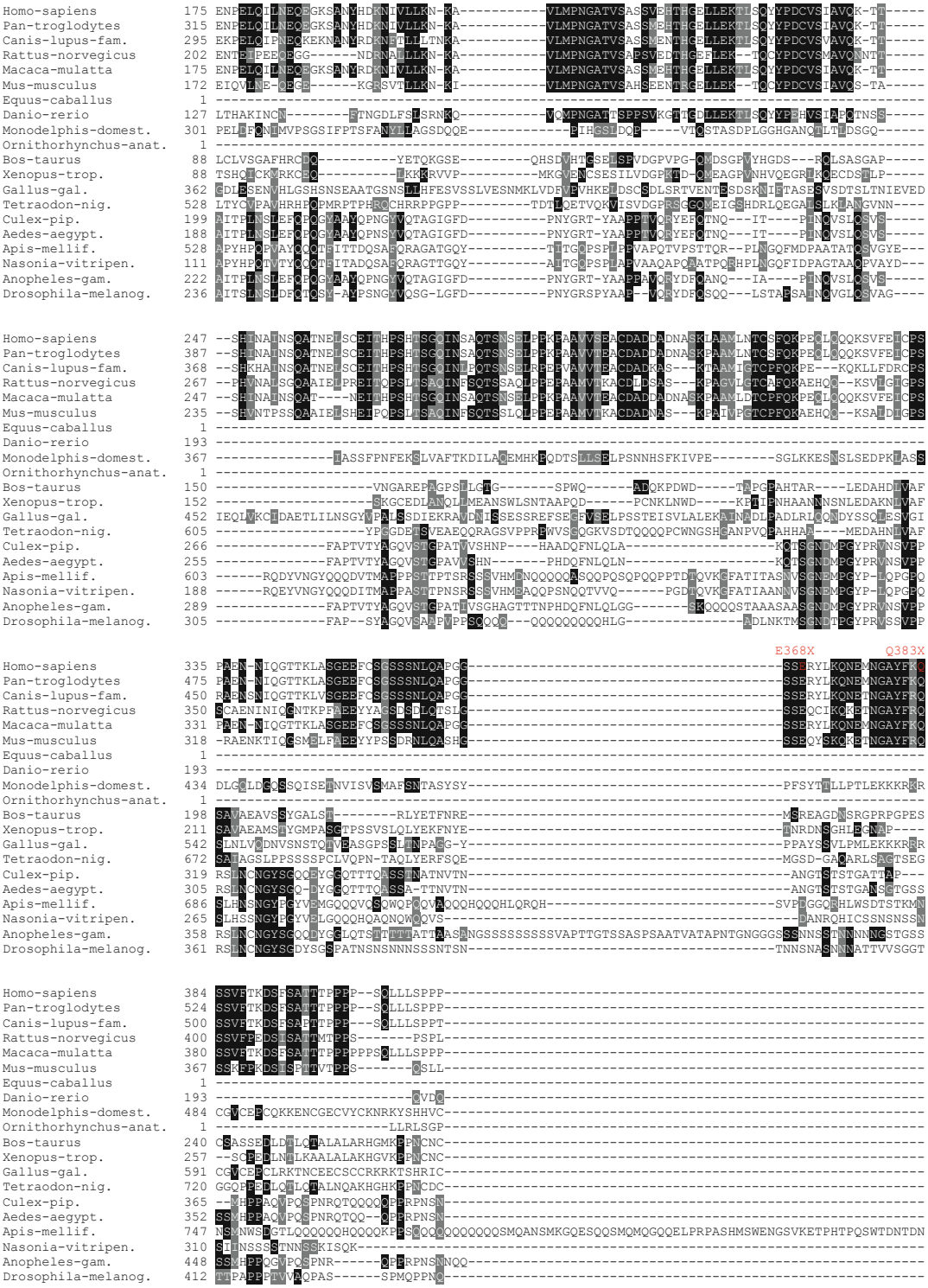


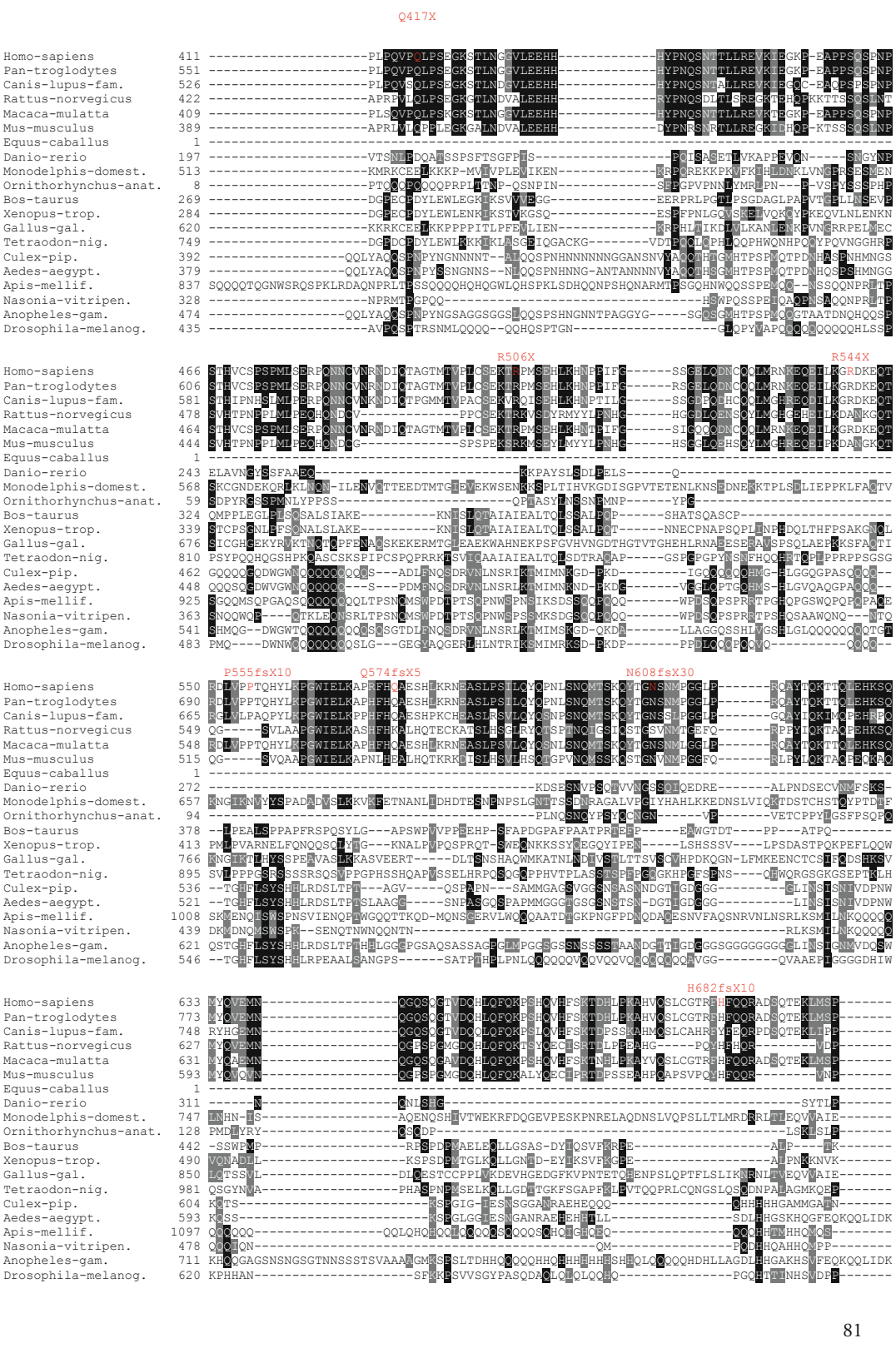
Supplementary Figure 2: TP53 mutation detection in a patient with UPD on chromosome 17p
(a) 250k SNP-array analysis of chromosome 17 of UPN 68 showing no copy number changes (in red). Green lines below the ideogram represent heterozygous SNP calls, which are nearly absent on the p-arm of chromosome 17, representing segmental UPD (indicated by a blue line). (b) Sequence analysis of the TP53 gene, which is located on 17p13.1, shows a homozygous deletion of a cytosine on position 723.

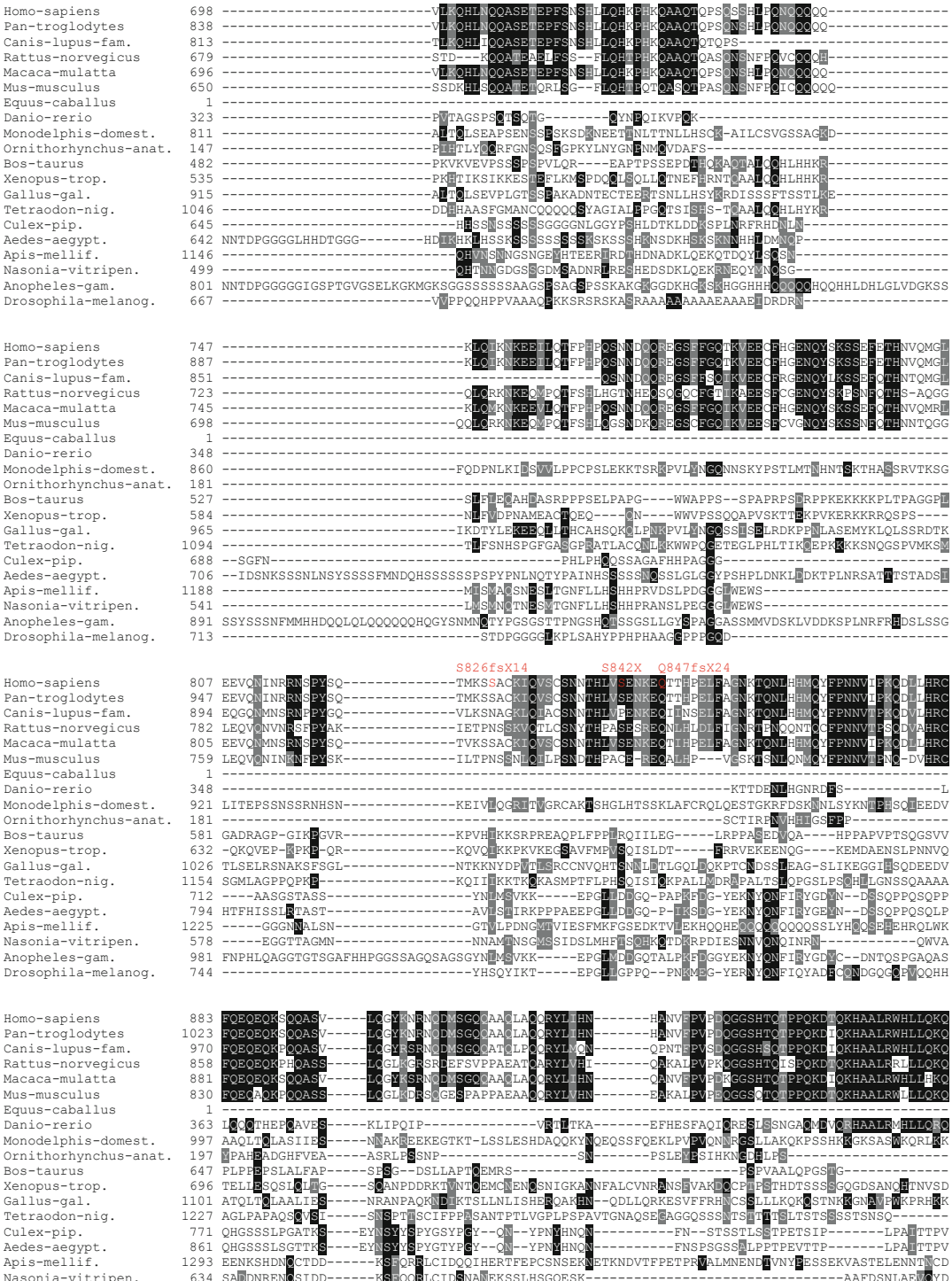
Supplementary Figure 3

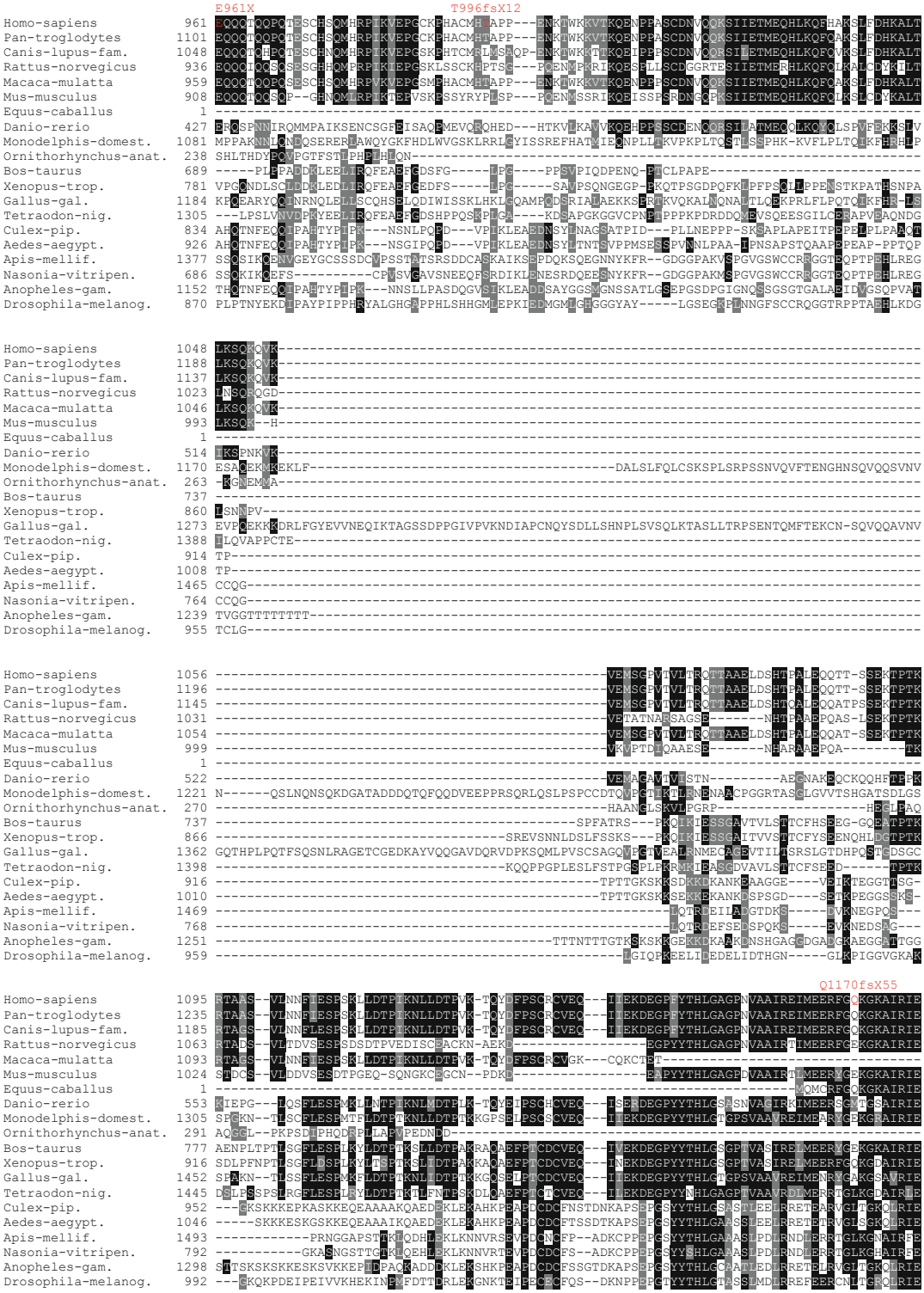



T164fsX5


E368X
Q383X


Q417X
R506X
R544X
P555fsX10
Q574fsX5
N608fsX30
H682fsX10
Homo-sapiens
Pan-troglodytes
Canis-lupus-fam.
Rattus-norvegicus
Macaca-mulatta
Mus-musculus
Equus-caballus
Danio-rerio
Monodelphis-domest.
Ornithorhynchus-anat.
Bos-taurus
Xenopus-trop.
Gallus-gal.
Tetraodon-nig.
Culex-pip.
Aedes-aegypt.
Apis-mellif.
Nasonia-vitripen.
Anopheles-gam.
Drosophila-melanog.

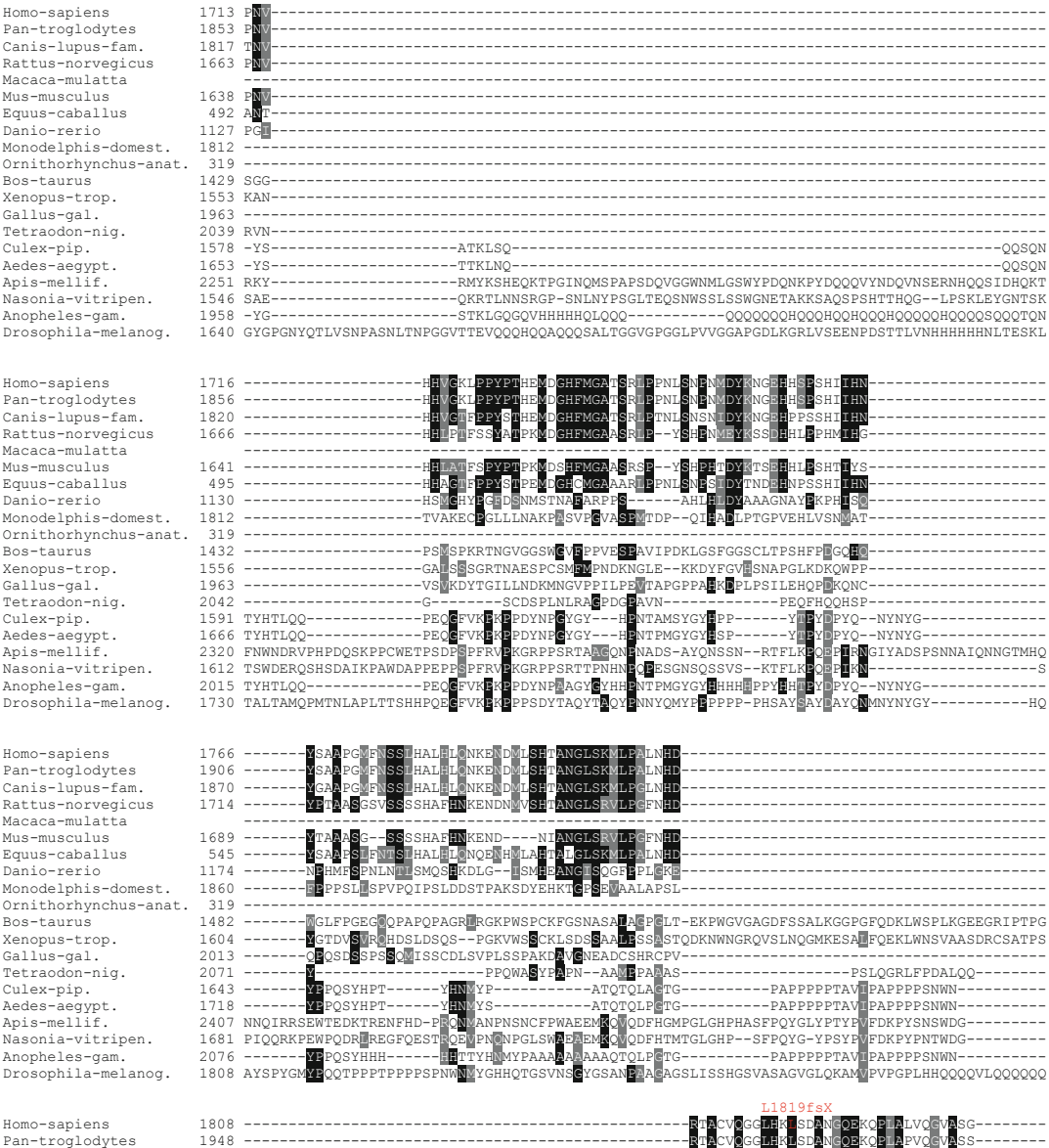

S826fsX14
S842X
Q847fsX24

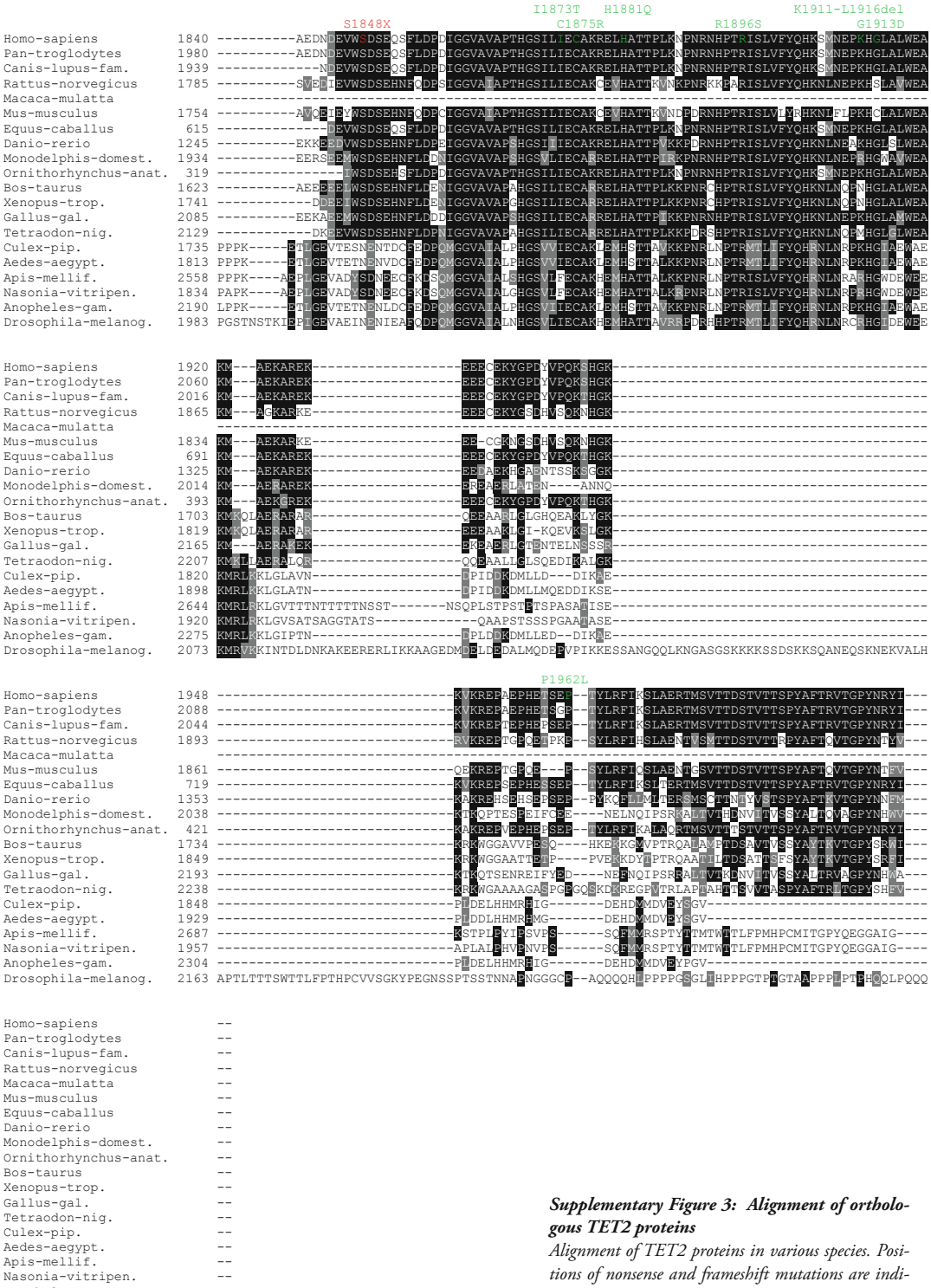

E961X
T996fsX12
Q1170fsX55
Homo-sapiens
Pan-troglodytes
Canis-lupus-fam.
Rattus-norvegicus
Macaca-mulatta
Mus-musculus
Equus-caballus
Danio-rerio
Monodelphis-domest.
Ornithorhynchus-anat.
Bos-taurus
Xenopus-trop.
Gallus-gal.
Tetraodon-nig.
Culex-pip.
Aedes-aegypt.
Apis-mellif.
Nasonia-vitripen.
Anopheles-gam.
Drosophila-melanog.

BOX 1

R1216X
L1240fsX2
R1261L
C1271fsX28
W1291R
C1271X
F1285del
L1398R
C1396W
S1424fsX23
BOX 1

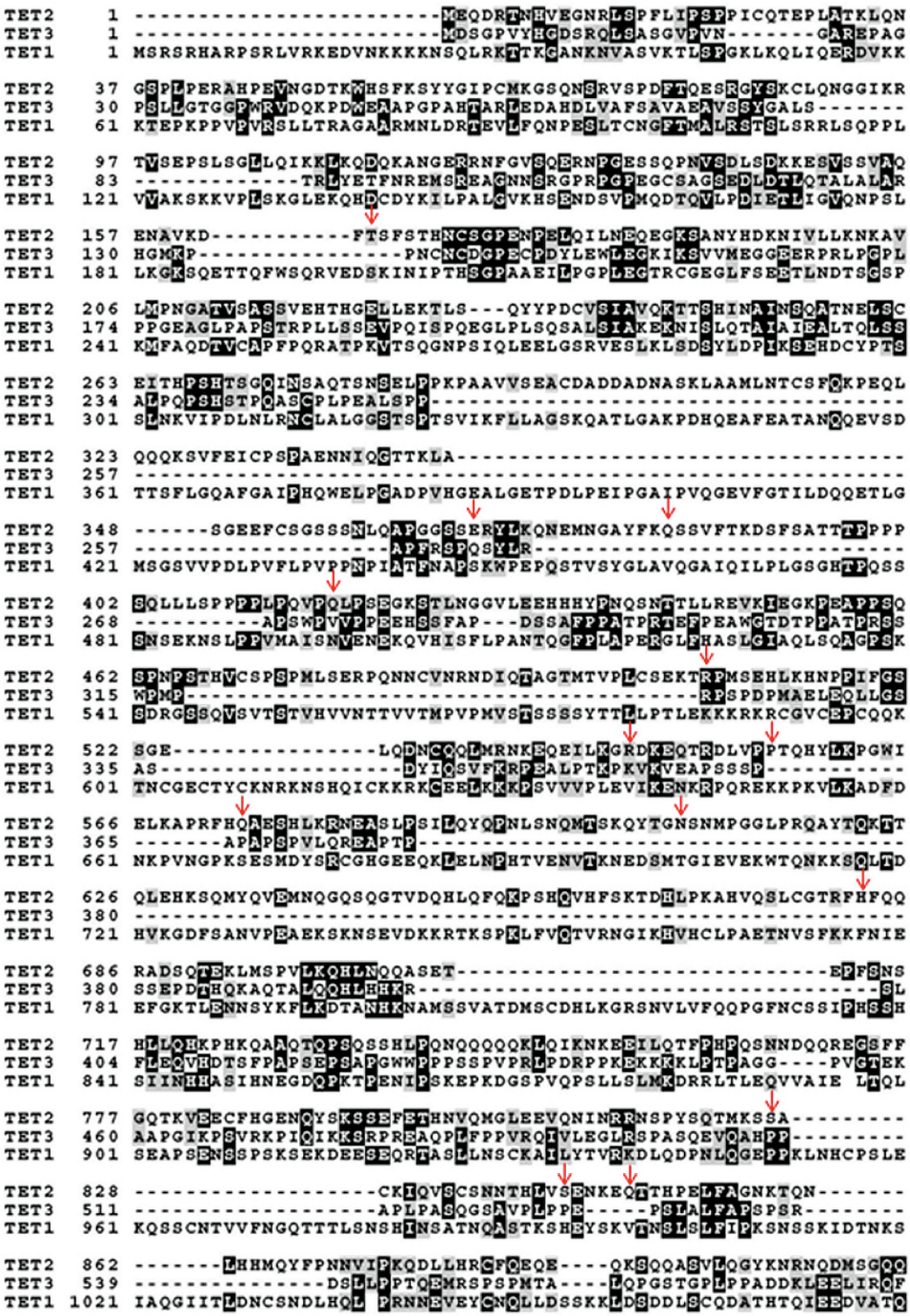

K1493fsX77
Homo-sapiens
Pan-troglodytes
Canis-lupus-fam.
Rattus-norvegicus
Macaca-mulatta
Mus-musculus
Equus-caballus
Danio-rerio
Monodelphis-domest.
Ornithorhynchus-anat.
Bos-taurus
Xenopus-trop.
Gallus-gal.
Tetraodon-nig.
Culex-pip.
Aedes-aegypt.
Apis-mellif.
Nasonia-vitripen.
Anopheles-gam.
Drosophila-melanog.

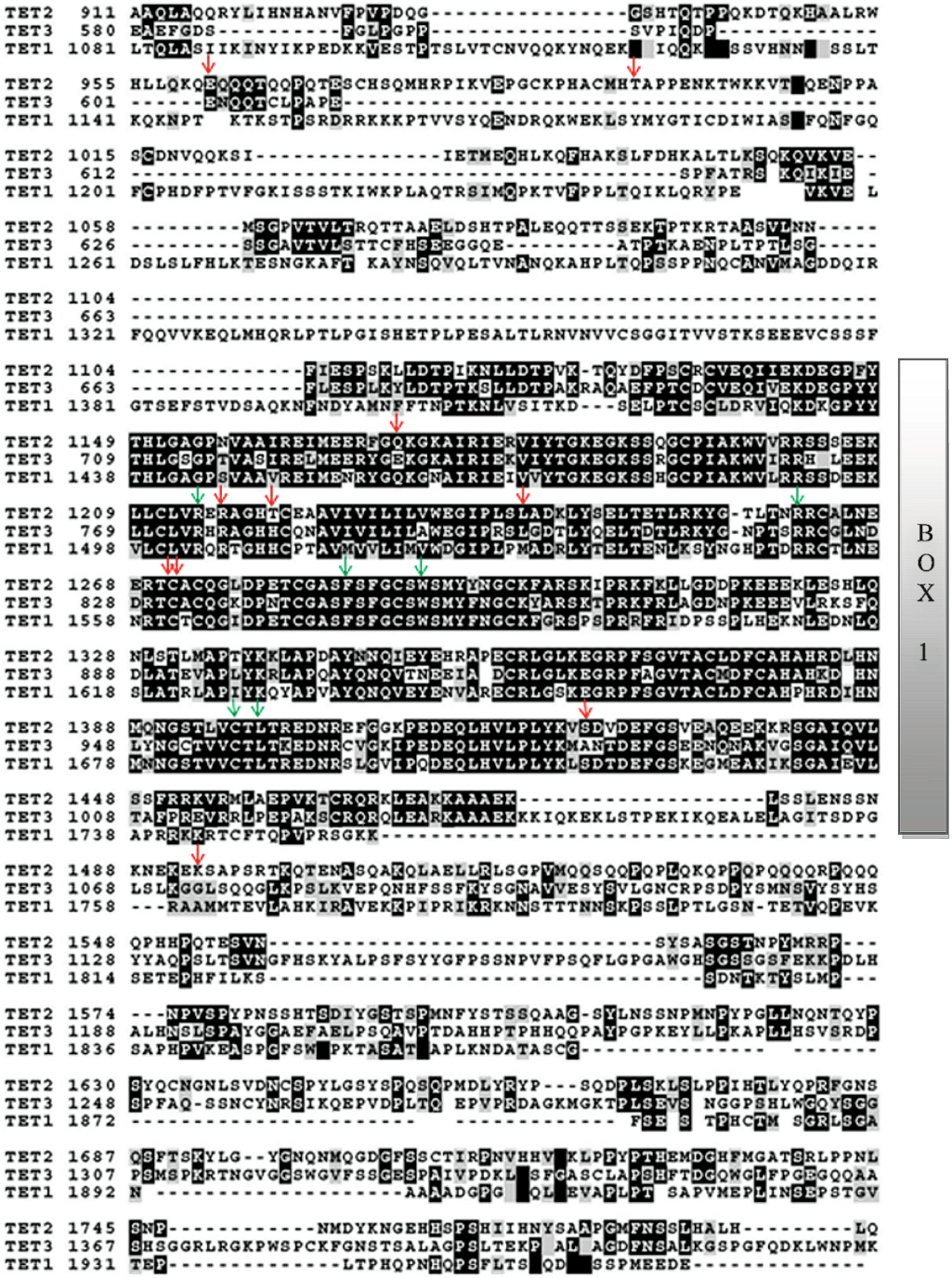

L1819fsX

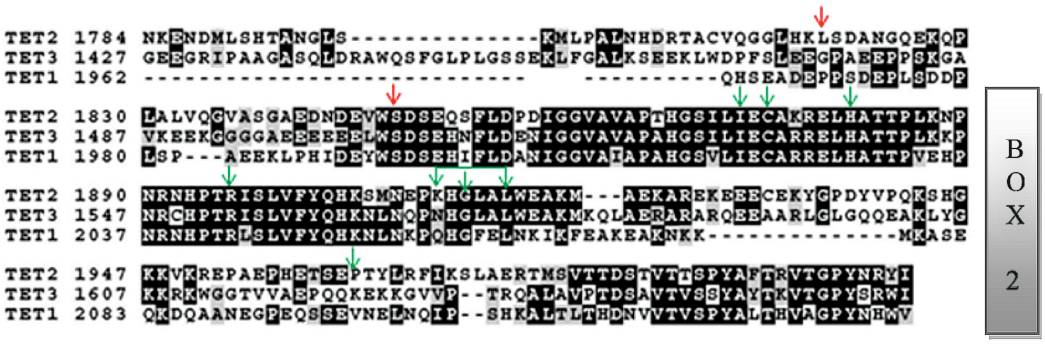


Supplementary Figure 3: Alignment of orthologous TET2 proteins

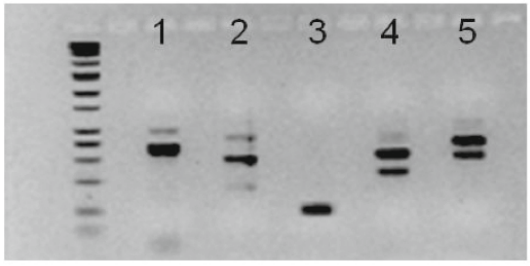
Alignment of TET2 proteins in various species. Positions of nonsense and frameshift mutations are indicated in red. Missense mutations and in-frame deletions are indicated in green.



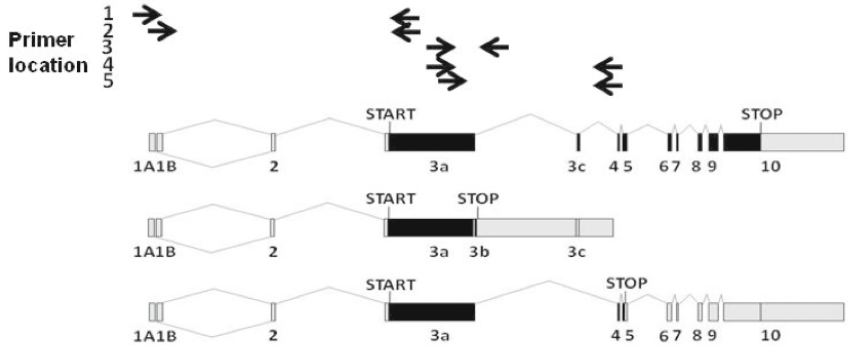

BOX 1



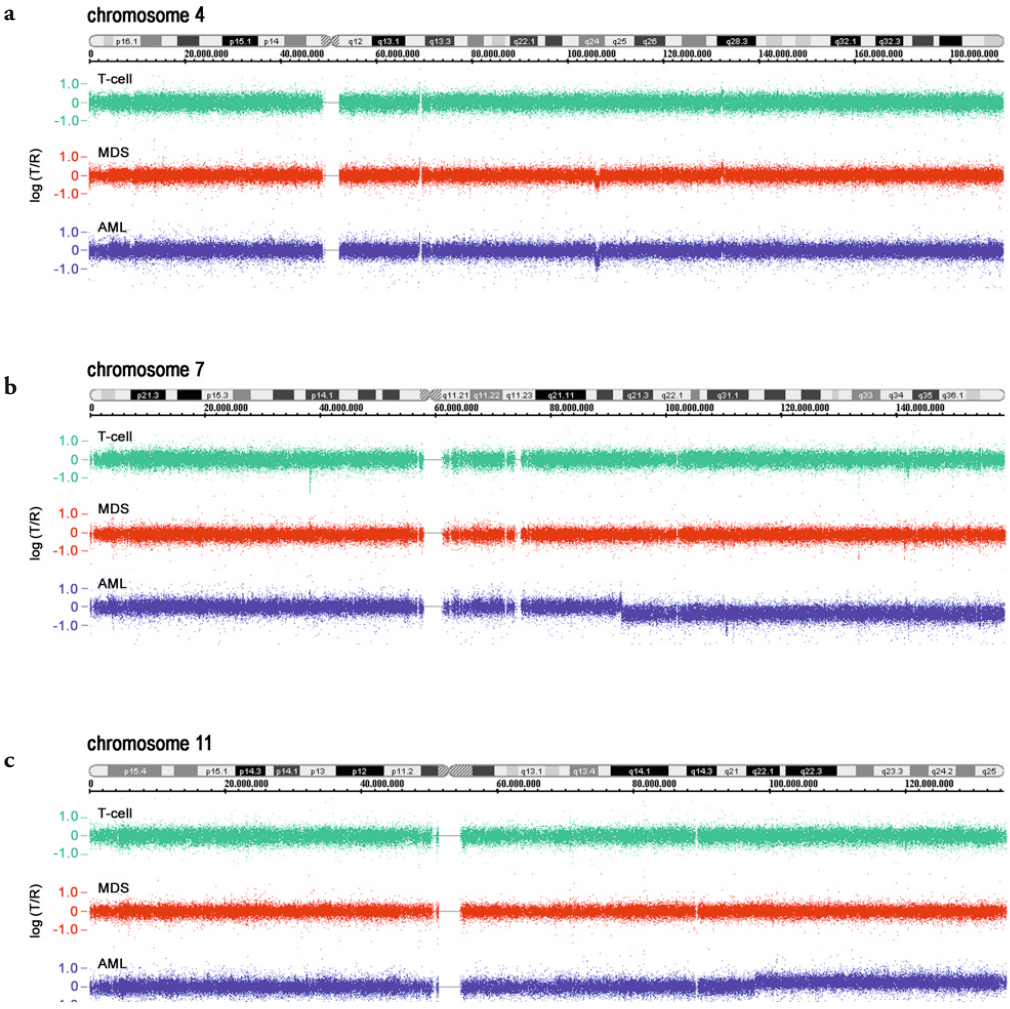
Supplementary Figure 4: Alignment of TET2 with homologues TET1 and TET3
Alignment of the TET2 protein with its two known homologues. Positions of nonsense and frameshift mutations are indicated in red. Missense mutations and in-frame deletions are indicated in green.



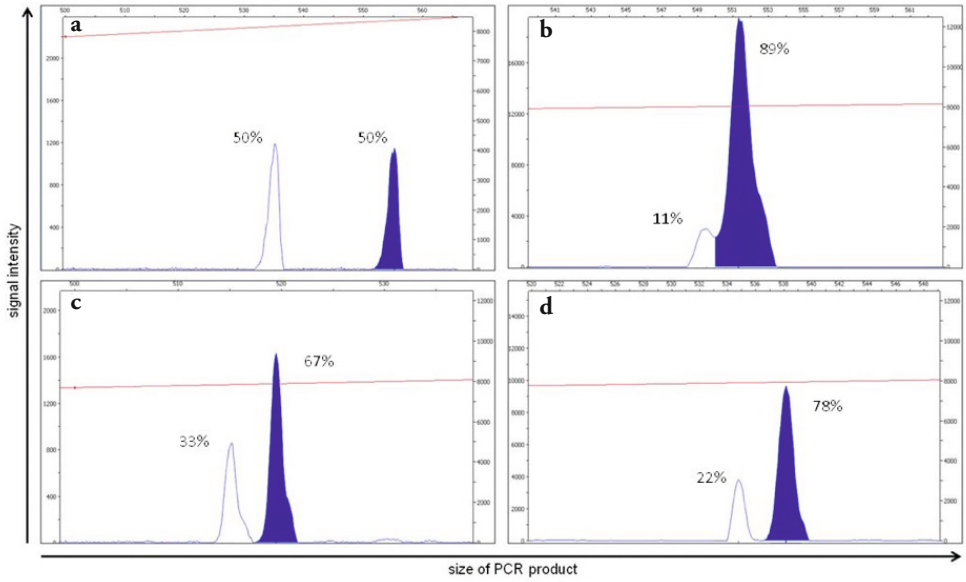
reaction	location primers (exon) forward/reverse	size products	exons present in sequenced PCR product
1	1A/3A	336	1A + 3A
		482	1A + 2 + 3A
2	1B/3A	293	1B + 3A
		439	1B + 2 + 3A
3	3A/3B	97	3A + 3B
4	3A/4	226	3A + 4
		317	3A + 3C + 4
5	3A/4	302	3A + 4
		393	3A + 3C + 4



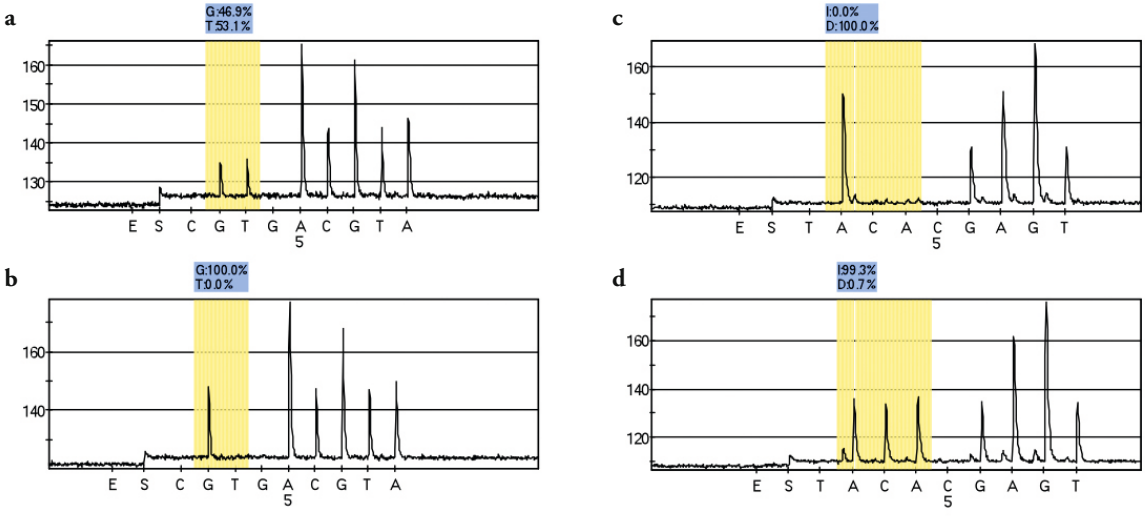
Supplementary Figure 5: Confirmation of TET2 splice-variants
Top left: agarose gel showing PCR products designed to cover the isoform-specific boundaries. The location of the primers is shown in the table (top right) and figure (bottom). The results of sequencing of the PCR products are shown in the table.



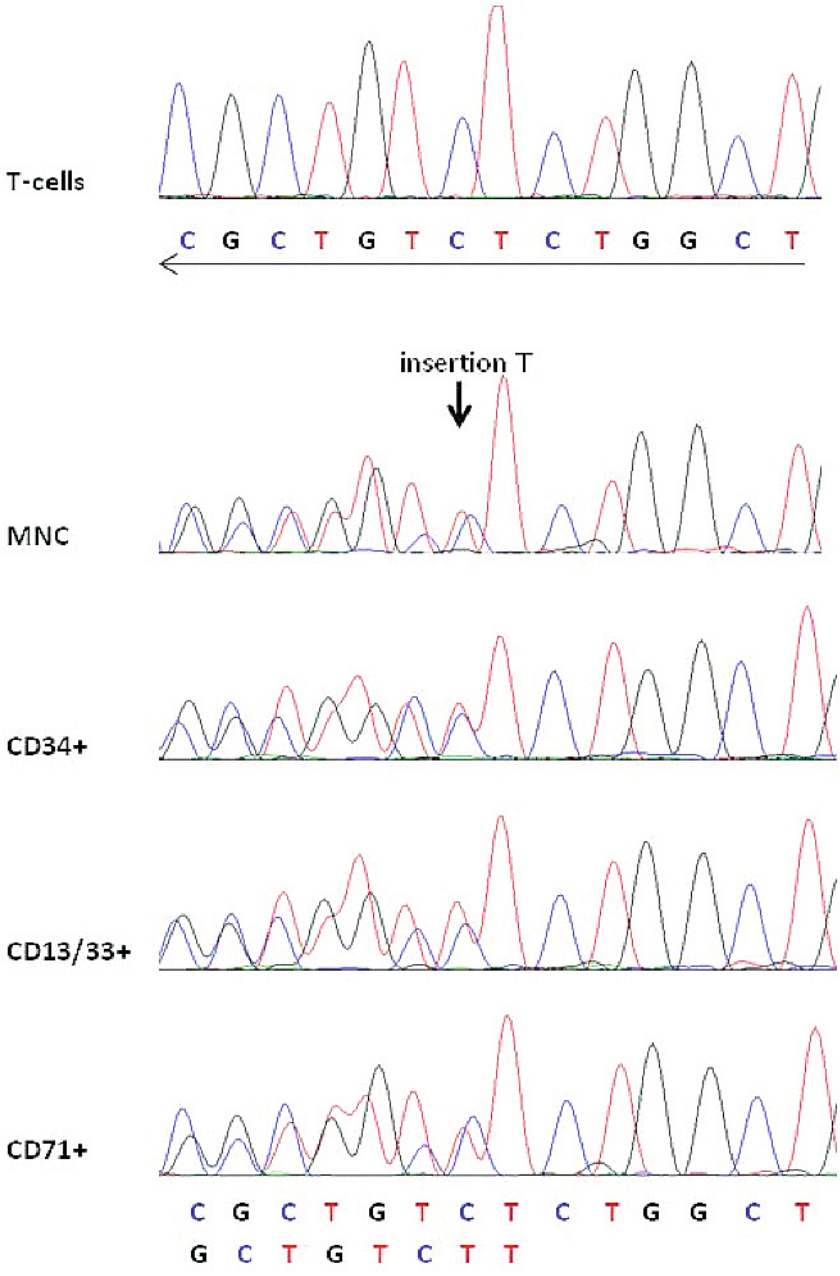
Supplementary Figure 6: Copy number aberrations in patient 44 before and after progression to AML
(a) SNP-array based analysis of chromosome 4 of patient 44 shows a microdeletion at 4q24 that is present both in the neoplastic MDS cells and in the leukemic blasts at the time AML was diagnosed. The microdeletion is absent in the T-cells.
(b and c) SNP-array based analysis of chromosomes 7 (b) and 11 (c) shows that the leukemic blasts of patient 44 show additional chromosomal aberrations, consisting of a 7q deletion and a (subclonal) 11q duplication, when compared to the MDS cells of the same patient.



Supplementary Figure 7: Determination of allelic burden using Genescan analysis
Results of Genescan analysis of 4 patients with a deletion in the TET2 gene of >1 basepairs. The blue peaks correspond with the wild type allele, the white peaks with the mutated allele. (a) Patient (UPN 101) with a 18 bp deletion in TET2. (b) UPN 103 showing a 2 bp deletion. This patient had a second mutation of TET2. (c) UPN 87 with 4 bp deletion. (d) UPN46 with a 3bp deletion. This patient carried a missense mutation of TET2 as well.



Supplementary Figure 8: Determination of allelic burden using pyrosequencing
Representative examples of pyrosequencing. (a) pyrosequencing of TET2 in UPN75 shows a G>T conversion (c.1102G>T) that is not present in the reference sample (b). (c) analysis of DNA from patient 74 shows a homozygous deletion of a cytosine (c.3508delC). The reference sample is shown for comparison (d)



Supplementary Figure 9: TET2 mutation detection in bone marrow subpopulations
Sequence analysis of TET2 was performed using DNA from T-cells, the mononuclear bone marrow cells and three hematopoietic subfractions of seven patients (Supplementary Table 6a). Sequence analysis of DNA from patient 77 is shown. The insertion of a thymine (c.3718_3719insT) is detected in all cell fractions, except T-cells.

Supplementary Table 1: Genomic copy number changes detected by 250k SNP-array analysis

**The column 'Reference' indicates which reference was used to exclude normal copy number variations. 1 = SNP-array analysis of individually hybridized DNA from 1015 healthy controls, 2= SNP-array analysis of DNA from pool of 10 healthy individuals hybridized simultaneously with SNP-arrays of respective MDS patients, 3= T-cells of the respective MDS patient. In addition, we compared our data with the data published in 'the database of genomic variants' as indicated in the methods section.*

UPN	FAB	WHO	IPSS	Cytogenetics	Chr
1	RAEB	RAEB1	int-1	46,XX	8
2	RAEBt	RAEB2	int-2	46,XY,t(12;22)(p13;q12)[5]/46,XY,idem, t(1;20) (q15;q12 [4]/47,XY,idem,+8[2]	4 12
3	RAEBt	RAEB2	high	47,XY,5,add(6)(p24),del(7)(q22),+8, add(12) (p13),+mar[2]/46,XY,-5, add(6)(p24), del(7)(q22),+8, add(12)(p13), -13,der(20)t(13;20)(q14;q12),+mar[8]	8 3 3 5 5 5 5 5 6 7 12 13 13 20 20 20
4	RAEB	RAEB1	int-1	46,XY	
5	RAEB-t	AML	high	46,XY, inv (7)(q22;q36)[9]/46,XY [11]	4 7 22
6	RAEB-t	AML	int-2	46,XX	8
7	RAEB-t	RAEB2	int-2	46~48, XY, -4, del(5)(q13q33), der(7) add (7)(p22) add(7)(q31), ?10(p11), tas(1;4;15)(p11;p11), -15, ?16, -21, +mar1, +mar2, +mar3, +mar4	6 15 16 21 21 21 21 4 5 6 7 10 10

** SNP-array analysis confirmed that well-known cytogenetic aberrations, such as loss of chromosome 7 and the co-occurrence of multiple cytogenetic defects correlated with a more aggressive phenotype. In this cohort, the newly defined copy number abnormalities did not significantly correlate with the established MDS subtypes.*

Cytoband	Size (Mb)	Type	start	end	Ref Seq genes (validated)	Genes	Reference
q22.1	0,07	Loss	96462846	96529372	0		1,2
q31.23	0,06	Loss	149518521	149575750	1	NR3C2	1,2
p13.2	0,11	Loss	11739760	11853840	1	ETV6	
p23.3-q24.3	146,08	Gain	180568	146263538	>50		1,2
p13-14.1	5,31	Loss	66376379	71682261	12		
q21.1	1,59	Loss	123309207	124896432	15		
p15.2-p15.1	1,98	Loss	13802952	15782509	6		
p11-q11.2	1,40	Loss	49596616	50994294	2	EMB, ISL1	
q14.3-q21.2	14,27	Loss	89576224	103846899	31		
q23.2-q31.3	15,15	Loss	126273797	141421684	>50		
q32-q34	16,84	Loss	144698251	161541603	>50		
p25-p24.3	11,03	Loss	119769	11151025	>50		
q21.13-q36.3	68,74	Loss	90060987	158798338	>50		
p13.31-p12.3	9,34	Loss	8007288	17344362	>50		
q12.3-q14.12	13,70	Loss	30646541	44347142	>50		
q14.3-q21.1	2,04	Loss	52477690	54520177	1	OLFM4	
p12.3-q11.22	20,71	Loss	7407565	28119554	>50		
q11.23-q13.12	9,13	Loss	34401989	43528383	>50		
q13.31-q13.32	0,61	Loss	54917455	55528558	5	BMP7, SPO11, RAE1, RBM38, CTCFL	
no aberrations							1,2
q21.23	0,29	Gain	84408893	84701659	6		1,2
q11.23	2,09	Gain	71680977	73771865	31		
q12.3	0,17	Gain	31045559	31212808	3	RFPL3, C22orf28, FBXO7	
q21.3	0,16	Loss	93074623	93233515	1	RUNX1T1	1,2,3
q24.1	0,81	Gain	140758746	141571579	0		1,2
q22.31-qter complex pattern	37,62	Gain	62570778	100192115	>50		
q21.1	0,49	Gain	22328428	22814209	0		
q21.2-3	6,31	Gain	23364758	29679365	20		
q21.3	0,33	Gain	29743331	30073388	1	GRIK1	
q22.13-qter	10,10	Gain	36796361	46894358	>50		
q13.1-q35.2	128,82	Loss	62352873	191167888	>50		
q14.3-q34	69,84	Loss	90859194	160694752	>50		
q24.1	0,44	Loss	142254945	142699855	2	NMBR, GPR126	
q31.1-q36.3	48,95	Loss	109843714	158798338	>50		
p12.1-q11.22	5,30	Loss	27103830	32408595	22		
q25.2-q25.3	2,11	Loss	114358392	116466398	12		

UPN	FAB	WHO	IPSS	Cytogenetics	Chr
8	RARS	RARS	int-1	46,XY	1 3
9	RA	RCMD	low	46,XX	
10	RAEBt	RAEB2	int-2	46,XX	
11	RARS	RCMD-RS	int-1	47,XX, +8 [6], 46,XX [4]	6 8 3
12	RA	RA	int-1	46,XY, -20, +mar	4 20 20 20 20
13	RAEB	RAEB2	high	47,XY, +11 [5] / 46,XY [15]	1 13 12
14	RA	RCMD	int-1	46,XX, t(1;21)(q21;q22)	
15	RAEBt	AML	high	46,XX	
16	RARS	RCMD-RS	int-1	46,XY del (20)(q12) [6]/45X, -Y, del(20)(q12) [3]/ 46XY [1]	20 20 21
17	RAEB	RAEB1	int-1	46,XY	
18	RAEB-t	RAEB2	int-2	46,XX	1
19	RARS	RCDM-RS	low	46,XY, possibly abberation 13 [in 4/32] en -Y [in 3/32]	X
20	RAEB	RAEB1	int-1	46,XY	1 6 6
21	RA	RCMD	int-1	46, XY	9 9 2 3
22	RAEBt	AML	high	47XY, +8 [5], 46XY [15]	
23	RA	RCMD	int-1	46,XX/46X, Xq-	11 X 17 17 X
24	RA	5q-	low	46,XX,del(5)(q13q33)	7 5
25	RAEB	RAEB2	int-2	46,XX	
26	RA/RAEB	RA/ RAEB1	Int-2	46,XX,del(7)(q22),del(20)(q11)[17]/46,XX [3]	7 20 21

Cytoband	Size (Mb)	Type	start	end	Ref Seq genes (validated)	Genes	Reference
p35.1	0,03	Loss	34043642	34076730	1	CSMD2	1,2
p14.2	0,10	Loss	61813257	61917627	1	PTPRG	
		no abberations					1,2
		no abberations					1,2
p25.2	0,04	Gain	2942543	2978804	1	NQO2	1,2
p23.3-q24.3	146,08	Gain	180568	146263538	>50		
q26.2	0,06	Loss	170273620	170330795	1	EVI1	
q12	0,22	Gain	56465465	56683501	2	EXOC1, CEP135	1,2,3
p11.1-q11.21	4,39	Gain	26027655	30419769	23		
q13.2-qter	12,17	Gain	50205838	62376958	>50		
p13-p11.1	25,92	Loss	17408	25938002	>50		
q11.21-q13.2	19,72	Loss	30455952	50173696	>50		
p31.3	0,07	Gain	62192315	62261604	1	INADL	1,2
q32.3	0,18	Gain	99477577	99659263	1	PCCA	
p12.3	0,08	Loss	16899069	16975264	0		
		no abberations					1,2
		no abberations					1,2
q11.22-23	0,81	Gain	33421667	34236327	13		1,2
q11.23-q13.2	14,78	Loss	35391249	50173696	>50		
q22.13-q22.2	0,48	Loss	38511815	38987776	2	KCNJ15, ERG	
		no abberations					1,2
q31.2	0,20	Loss	189824778	190021009	0		1,2
q27.1	0,42	Gain	139482978	139903522	1	CDR1	1,2
p35.3	0,22	Gain	29023832	29244086	0		1,2,3
q15	0,13	Gain	90428707	90555860	0		
q16.1	0,48	Gain	96718690	97201861	3	FUT9, KIAA0776, FHL5	
q22.1	0,45	Gain	89747998	90202303	2	CCRK, SPIN1	1,2
q22.31	0,58	Gain	93612061	94193550	7		
q35	0,07	Loss	217399487	217467761	1	TNP1	
p24.3	0,04	Loss	17003231	17045586	1	PLCL2	
		no abberations					1,2
q12.2	0,10	Gain	60123089	60222990	1	C11orf64	1,2
pter-p11.1	57,80	Gain	142664	58075602	>50		
q25.1-q25.3	1,91	Loss	71568536	73478189	22		
q25.2	0,22	Loss	72010995	72229750	6		
q11.2-q28	91,75	Loss	62794454	154500683	>50		
q36.1-2	0,34	Gain	151950636	152292559	2	XRCC2, ACTR3B	1,2
q14.3-q33.3	72,52	Loss	83559251	156083322	>50		
		no abberations					1,2
q21.3-36.2	59,06	Loss	94297190	153359077	>50		1,2
q11.21-q13.2	18,41	Loss	31058802	49469644	>50		
q22.12	0,15	Loss	35234754	35386104	1	RUNX1	

UPN	FAB	WHO	IPSS	Cytogenetics	Chr
27	RARS	RARS	int-1	47,XY, +21 [7]/46XY [3]	9 21 8 15 20
28	RAEB	RAEB1	int-2	46,XX, +1, der ((1;7)(q10;p10)	1 7
29	RAEB	RAEB2	int-2	46,XY	9
30	RAEB	RAEB2	int-2	46,XY	6 13
31	RARS	RARS	low	46,XY	
32	RAEB	RAEB2	high	47,XY, +8 [7],/47XY, +21 [8]/46XY [5]	3 8 21 1 13 X
33	RAEBt	RAEB2	int-1	46XY, ?(17q) [7]/46XY [3]	
34	RAEB-t	RAEB2/ AML	int-2	46,XX	1 2 7 12 22 22 2 10 18
35	RA	RCMD	int-1	47,XY, +21, del(20)(q11)	14 10 20
36	RARS	RCMD-RS	low	46,XY	
37	RAEBt	AML	int-2	46,XY	

Cytoband	Size (Mb)	Type	start	end	Ref Seq genes (validated)	Genes	Reference
p13.2	0,23	Gain	36489448	36717652	0		1,2
q22.11	0,24	Gain	34027879	34268175	2	ITSN1, ATP50	
q22.1	0,12	Loss	93733795	93858178	0		
q22.2	0,13	Loss	57997597	58122959	1	FOXB1	
p11.23	0,05	Loss	19928024	19979632	3	RIN2, NAT5, CRNKL1	
q12-q44	103,85	Gain	142756696	247110269	>50		1,2
q11.21-q36.3	97,06	Loss	61740813	158798338	>50		
p24.3	1,14	Gain	1344499	2483292	1	SMARCA2	1,2
p22.3	0,05	Loss	15515724	15564163	1	JARID2	1,2
q21.31	0,03	Loss	63497560	63530806	0		
no abberations							1,2
p14.3	0,58	Gain	57434952	58015066	6		1,2
p23.2-q24.21	122,78	Gain	6047363	128823871	>50		
q21.1-q22.3	37,01	Gain	9887804	46894358	>50		
q41	0,00	Loss	213927087	213928502	1	USH2A	
q32.3	0,02	Loss	99949768	99973880	1	PCCA	
q26.2-q26.3	1,30	Loss	133304184	134603832	12		
no abberations							1,2,3
q21.3	0,58	Gain	151880407	152463750	12		1,2
p16.2	0,29	Gain	53968471	54257611	2	PSME4, ACYP2	
p22.1	1,06	Gain	5662651	6720219	17		
q24.31	2,17	Gain	119533318	121719778	31		
q12.2	0,59	Gain	28135407	28723544	10		
q12.2-3	1,90	Gain	29316172	31212808	27		
p16.3	0,17	Loss	52128333	52295286	0		
q11.23	0,01	Loss	49581707	49590614	1	WDFY4	
q22.1	0,05	Loss	63985304	64034323	0		
q21.3	0,18	Gain	49624023	49807419	3	c14orf138, SOS2, L2HGDH	1,2
q21.3	0,14	Loss	68064862	68201848	1	CTNNA3	
q11.21-q13.2	18,82	Loss	31204465	50029445	>50		
no abberations							1,2
no abberations							1,2

UPN	FAB	WHO	IPSS	Cytogenetics	Chr
38	RAEB	RAEB1	int-2	46,XX, ?del(12)(q23q24), del(20)(q11) [7]/46,XX, idem, del(5)(q15) [2]/ 46,XX [2]	2 4 6 6 11 12 13 X
39	RAEB-t	RAEB2	int-1	46,XY	1 8
40	RAEB	RAEB2	high	46,XY, t(3;16)(q26;q2?3) [2]/ 46,XY [8]	7 10 12
41	RA	RARS	int-1	47,XY, +19 [8]/46,XY [2]	19 X
42	RAEB	RAEB1	int-1	46,XY	
43	RA	RCMD	int-2	46,XX, del (5)(q13q33) [1]/45,XX, del(5)(q13q33), -7 [5]	
44	RAEB	RAEB2	int-2	46,XY	8 9 18 4 7
45	RAEB	RAEB1	int-2	46,XX,del(5)(q13;q33),t(X;16)(p11;q24), inv (12)(p12q23)	5 11
46	RA	RCMD	low	46,XX	
47	RAEBt	AML	int-1	46,XY	9 11 21
48	RAEB	RAEB2	int-2	46,XY	3
49	RARS	RCMD-RS	int-1	46,XX	
50	RA	RCMD	low	46,XY	12
52	RAEB	RAEB1	int-1	46,XY, del (9)(q22q32)	9 13
53	RAEB	RAEB1	int-2	42,X, -Y,-5,-7,-17,-18,+mar1[5]/ 43,idem,+mar2[2]/46,XY[3]	13 5 5 7 18

Cytoband	Size (Mb)	Type	start	end	Ref Seq genes (validated)	Genes	Reference
p23.3	1,13	Gain	24121646	25249516	9		1,2
p15.33	0,26	Loss	14553996	14815436	1	CPEB2	
p22.3	0,02	Loss	15515724	15532723	1	JARID2	
p21.1	0,13	Loss	45520635	45645653	1	RUNX2	
p15.3	0,12	Loss	11346019	11463667	1	GALTNL4	
p12.3	0,08	Loss	16899069	16975264	0		
q21.33	0,06	Loss	69587308	69643614	1	ATXN8OS	
q13.1	0,17	Loss	68747848	68914019	1	EDA	
q21.3	0,38	Gain	152088568	152463750	8		1,2
q24.21	0,13	Gain	130597259	130723928	0		
p22.2	0,20	Gain	4298964	4495687	0		1,2
p11.23	0,43	Gain	30473297	30902186	3	MTPAP, MAP3K8, LOC729668	
q23.2	0,06	Loss	101321326	101380604	1	IGF1	
p13.3-q13.43	63,52	Gain	212033	63731511	>50		1,2
p21.1	0,63	Gain	35722446	36348491	2	CXorf22, CXorf30	
		no abberations					1,2
		no abberations					1,2
q12.1	0,18	Gain	56860110	57045025	2	TGS1, LYN	1,2
p24.1	0,66	Gain	6212553	6868215	5	IL33, GLDC, UHRF2, TP-D52L3, JMJD2C	
q21.1	0,43	Gain	46524501	46951934	4	MRO, ME2, ELAC1, SMAD4	
q24.23	0,83	Loss	105908963	106740191	2	TET2, PPA2	
p22.3-q36.3	158,46	Loss	141322	158798338	>50		
q15-q33.2	58,24	Loss	96824371	155068532	>50		1,2
q25	0,05	Loss	130408303	130457358	0		
		no abberations					1,2
q21.33	0,56	Gain	87662476	88225190	4	MAK10, GOLM1, ISCA1, ZCCHC6	1,2
q12.1	0,31	Gain	57350416	57662037	2	OR6Q1, OR9I1	
q22.12	0,15	Loss	35156070	35308597	1	RUNX1	
p24.3	0,08	Loss	17081031	17157701	1	PLCL2	1,2
		no abberations					1,2
q23.2	0,04	Loss	101338462	101380604	1	IGF1	1,2
q21.2-q31.1	23,26	Loss	78591021	101851030	>50		1,2
q31.3	0,01	Loss	93195621	93202489	1	GPC6	
q32.1	0,23	Gain	94686683	94921466	2	ABCC4, CLDN10	1,2,3
p15.31-15.2	3,27	Loss	8822738	12088080	8		
q11.1-q35.3	131,03	Loss	49596616	180629495	>50		
p22.3-q36.3	158,46	Loss	141322	158798338	>50		
p11.32-q23	75,91	Loss	210071	76115554	>50		

UPN	FAB	WHO	IPSS	Cytogenetics	Chr
54	RAEB	RAEB2	high	46,XX, t(6;9)(p23;q34)	18 18
55	RA	RA	int-1	46,XX, t(3;3)(q21;q26) [6]/ 46XX [4]	2
56	RA	RCMD	int-1	46,XX	1
57	RAEB	RAEB2	Int-2	46,XY	12
58	RAEB	RAEB2	high	46,XX, del (1)(q11), del(5)(q14q34), -7, -18, +mar1, +mar2 [10], 55,XX, idem, +del(5)(q14q34), +8, +9, +10, +11, +14, +15, +21, +22 [9]/ 46,XX [1]	1 22 1 1 1 1 5 7 7 18
59	RAEB	RAEB1	int-2	45,XX, -7 [9], 46XX [1]	1 7
60	RARS	RCMD-RS	low	46,XY	3 12
61	RARS	RCMD-RS	low	46,XY	
62	RARS	RCMD-RS	low	46,XX	2 2
63	RARS	RCMD-RS	low	46,XY	1 9
64	RAEB	RAEB2	high	43-46,?Y,add (X)(q28),-5,-6,del(7)(q22?), -10,-11,-12,-17,+15,+21,+mar1-3[16]/46,XY[4]	5 5 5 5 6 6 7 10 12 12 12 17

Cytoband	Size (Mb)	Type	start	end	Ref Seq genes (validated)	Genes	Reference
q12.1	0,13	Loss	29450139	29578054	1	ASXL3	1,2
q22.1	0,03	Loss	64003187	64034323	0		
q24.3	0,11	Loss	166903186	167012202	1	SCN7A	1,2
p21.1	0,10	Loss	105679389	105776622	0		1,2
q23.2	0,03	Loss	101321326	101351175	1	IGF1	1,2
q22-23.1	1,33	Gain	153918705	155250725	33		1,2
q12.2	0,26	Gain	28025929	28290335	8		
p31.2	0,93	Loss	65709653	66640400	2	LEPR, PDE4B	
p31.2-p22.2	21,38	Loss	67182626	88561025	>50		
p22.1-22.3	2,82	Loss	93955123	96779720	10		
p13.2-3	2,59	Loss	110106932	112699113	29		
q21.3-q35.2	68,00	Loss	107276507	175279396	>50		
p15.3	0,43	Loss	22010967	22445643	1	RAPGEF5	
p12.3-q36.3	109,52	Loss	49280157	158798338	>50		
q11.2-q23	55,77	Loss	20348828	76115554	>50		
q41	0,03	Loss	213927087	213954109	1	USH2A	1,2
p22.3-q36.3	158,46	Loss	141322	158798338	>50		
q13.12-13	0,38	Gain	109052445	109427768	4	LOC151658, IFT57, LOC285205, CD47	1,2
q21.32	0,03	Loss	87344571	87370350	0		
		no abberations					1,2
p22.3	0,81	Gain	32134203	32942048	4	SPAST, NLRC4, YIPF4, BIRC6	1,2
q23.3	0,20	Loss	154085999	154290496	0		
p35.1	0,01	Loss	34056481	34067461	1	CSMD2	1,2
q31.1	1,93	Loss	103835445	105762025	1	CYLC2	
p13.2-13.1	2,07	Loss	37334638	39400231	8		1,2,3
q12.1-q12.3	3,34	Loss	60882776	64218736	9		
q12.3-q13.2	5,76	Loss	65200747	70959114	29		
q13.3-q35.3	105,43	Loss	75197159	180629495	>50		
p22.3	5,04	Loss	15481631	20522665	15		
p22.3	1,28	Loss	22055196	23338700	0		
q11.21-q36.3	97,06	Loss	61740813	158798338	>50		
q11.21-11.22	7,17	Loss	42155347	49321754	40		
p13.33-q14.1	61,06	Loss	50446	61106231			
q21.31-q23.1	18,24	Loss	81443995	99681334	>50		
q23.3-q24.33	25,95	Loss	106454871	132287718	>50		
p13.3-q11.1	22,01	Loss	18901	22029237	>50		

UPN	FAB	WHO	IPSS	Cytogenetics	Chr
65	RARS	RARS	low	46,XX	2
					9
					11
					11
					20
					22
					1
					16
					18
66	RA	RCMD	int-1	47,XY, +8 [5]/ 46,XY [5]	8
					5
					12
67	RARS	RCMD-RS	low	46,XY	
68	RAEBt	RAEB2	high	46-48 XY, -5,-7, t(12;12)(p13;q22), add (21)(q22), -22x2, +r1, +r2, +r2, +mar1, +mar2, +mar3, +mar3 [cp9]/46,XY [1]	3
					21
					21
					21
					21
					22
					5
					6
					6
					7
					7
					7
					7
					12

Cytoband	Size (Mb)	Type	start	end	Ref Seq genes (validated)	Genes	Reference
q13	0,43	Gain	112666184	113098605	4	ZC3H8, ZC3H6, TTL, POLR1B	1,2
q21.11	0,14	Gain	70861066	70996190	2	FXN, TJP2	
q13.4	0,29	Gain	73188293	73477649	5	MRPL48, CH-CHD8, UCP2, UCP3, C2CD3	
q14.1-2	0,22	Gain	85272986	85497725	1	PICALM	
q11.22-23	2,59	Gain	31511054	34103073	38		
q13.1-2	0,96	Gain	38930433	39888178	10		
q41	0,03	Loss	213927087	213959926	1	USH2A	
q23.3	0,06	Loss	80717166	80780421	1	MPHOSPH6	
q12.3	0,08	Loss	39201389	39276981	0		
p23.2-1	0,14	Gain	6116508	6257591	1	MCPH1	1,2,3
q21.1	0,07	Loss	102105029	102170530	0		
p13.2	0,17	Loss	11771243	11939631	1	ETV6	
		no abberations					1,2,3
q13.13	0,60	Gain	110153292	110751304	4	GUCA1C, MORC1, C3orf66, DPPA4	1,2
q22.13-2	2,53	Gain	36927512	39459104	13		
q22.2	0,62	Gain	40566752	41185373	1	DSCAM	
q22.2-3	0,89	Gain	41215523	42109613	10		
q22.3	3,63	Gain	42159278	45789927	>50		
complex pattern							
p12-qter	131,03	Loss	49596616	180629495	>50		
q21	2,24	Loss	106097724	108338935	10		
q21	0,14	Loss	109064223	109206411	1	FOXO3	
q11.22-q21.11	11,71	Loss	68428002	80136823	>50		
q21.11-q21.12	2,11	Loss	84526794	86638287	4	SEMA3D, GRM3, KIAA1324L, DMTF	
q21.12-q21.3	9,96	Loss	86981089	96941847	48		
q22.1-q36.3	59,50	Loss	99298043	158798338	>50		
p12.3	2,10	Loss	15551848	17655985	6		

UPN	FAB	WHO	IPSS	Cytogenetics	Chr
69	RAEB	RAEB1	int-2	58,XY, +1, +2, +8, +9, +10, +11, +13, +14, +20, +21, +21, add (22)(p13), +mar1 [10]	1
					2
					8
					9
					9
					10
					11
					13
					14
					20
					21
					22
					16
71	RAEB	RAEB 2	int-2	46,XY	7
					8
					10
					10
					11
72	RAEB	RAEB 1	int-1	46,XY	1
73	RAEB	RAEB 1	int-1	47, XX, +8 [7]/46,XX [3]	8
					8
					2
74	RAEBt	AML	high	46,XY	15
					18
75	RAEB	RAEB1	int-1	46,XY	
76	RAEB t	AML	high	45,XX, del(5), -7	7
					8
					5
					5
					7
					7
					8
					12
					12
					16
					16
					20
77	RAEB	RAEB 1	int-1	46,XY	4
78	RARS	RCMD-RS	low	46,XX	
79	RARS	RCMD-RS	int-1	46,XY	7

Cytoband	Size (Mb)	Type	start	end	Ref Seq genes (validated)	Genes	Reference
p36.33-q44	243,22	Gain	2095738	247110269	>50		1,2
p25.3-q37.3	242,69	Gain	24049	242650580	>50		
p23.3-q24.3	146,08	Gain	180568	146263538	>50		
pter-q31.1	103,17	Gain	30910	105160491	>50		
q31.1-qter	34,63	Gain	105638843	140147760	>50		
p15.3-q26.13	126,80	Gain	148946	126946025	>50		
p15.5-q25	134,24	Gain	201447	134439182	>50		
q11-q34	96,13	Gain	17960319	114092980	>50		
q11.2-q32.33	86,85	Gain	19502641	106356482	>50		
p13-q13.33	62,36	Gain	17408	62376958	>50		
p11.2-q22.3	36,63	Gain	9887804	46522771	>50		
q11.1-q13.33	35,02	Gain	14441016	49522492	>50		
p13.2	0,10	Loss	6974942	7073249	1	A2BP1	
p12.3	0,03	Loss	48496055	48527687	1	ABCA13	1,2
q24.22	0,07	Loss	131905912	131972316	1	ADCY8	
p14	0,02	Loss	11132318	11152680	1	CUGBP2	
q23.31	0,38	Loss	91579028	91962828	0		
p14.1	0,04	Loss	30699318	30737507	0		
p31.1	0,05	Loss	82832295	82881647	0		1,2
p23.3-p12	35,94	Gain	180568	36115976	>50		1,2
p12-q24.3	110,07	Gain	36196622	146263538	>50		
p16.3	0,12	Loss	50817046	50934328	1	NRXN1	
q21.1	0,06	Loss	45864214	45920576	0		1,2
q12.1	0,05	Loss	29473233	29527498	1	ASXL3	
		no abberations					1,2
p22.3-p22.2	1,32	Gain	1969259	3293525	10		1,2
p11.22-q24.3	106,64	Gain	39624983	146263538	>50		
p13.2-p13.1	1,39	Loss	38183243	39568994	7		
q15-q34	70,62	Loss	94248164	164864377	>50		
p21.3	3,10	Loss	7501906	10605336	6		
p21.2-q36.3	143,63	Loss	15171632	158798338	>50		
p23.3-p22	16,28	Loss	1134406	17415901	>50		
p13.2-p12.1	13,44	Loss	11892519	25336389	>50		
p12.1-p11.1	8,69	Loss	25362747	34051454	30		
q12.1-q22.1	16,41	Loss	50943957	67352875	>50		
q22.2-q24.3	18,12	Loss	70572907	88690776	>50		
p13-p11.23	18,63	Loss	17408	18645665	>50		
q31.3	0,82	Gain	154498695	155319054	5	MND1, SFRP2, TLR2, KIAA0922, RNF175	1,2
		no abberations					1,2
p21.2-p21.1	0,47	Gain	14762273	15229190	1	DGKB	1,2

UPN	FAB	WHO	IPSS	Cytogenetics	Chr
80	RARS	RARS	int-1	47,XY, +8 [4]/46,XY [6]	8 11 13 13
81	RA	RCMD	Int-1	46,XX	1
83	RA	MDS-U	low	46,XY	11
84	RA	RA	low	46,XX	
85	RA	RA	Int-1	46, XY	11
86	RA	RA	low	46,XY	20
87	RA	RA	low	46,XY	7 14
88	RA	RA	low	46,XX	14
89	RA	RA	low	46,XY	4 10
90	RA	RA	low	46,XY	3 3
91	RA	RA	low	46,XX	
92	RA	RCMD	low	46, XY	18
93	RA	RCMD	Int-1	46,XY	13 14
94	RA	RCMD	Int-1	46, XX	9
96	RA	RCMD	low	46,XX	
97	RA	RCMD	Int-1	46,XY	21
98	RA	RCMD	low	46,XY	3 18
99	RA	RCMD	low	46,XY	15 2 12 18
100	RA	RCMD	low	46, XY	4
102	RA	RCMD	low	46, XY	14
103	RAEB	RAEB1	int-1	46,XX	
104	RAEBt	AML	high	43-48, XY, -5 [14], +4 [5], -4 [2], +18 [5], t(11;17) [11], t(21;21) [2]	5 7 8 14 21 6 7 15 15 17 22
105	RAEBt	RAEB2	int-2	46, XY	14

Cytoband	Size (Mb)	Type	start	end	Ref Seq genes (validated)	Genes	Reference
p23.3-q24.3	146,05	Gain	180568	146225933	>50		1,2
p11.2-q14.1	32,55	Gain	44984291	77533005	>50		
q14.3-q31.3	41,25	Loss	52538263	93786901	43		
q33.1-q34	11,73	Loss	102330199	114063361	32		
q25.2	0,02	Loss	178024667	178040788	0		1,2
q22.3	0,24	Gain	107129408	107368940	1	SLC35F2	1,2
		no abberations					1,2
q22.3	0,05	Gain	107198330	107252235	1	SLC35F2	1,2
p11.21	0,12	Loss	25277915	25399180	3	ABHD12, GINS1, NLP	1,2
q36.1	0,13	Loss	148058806	148191991	2	CUL1, EZH2	1,2
q23.3	0,04	Loss	65268892	65309210	1	FUT8	
q32.32	0,34	Gain	102137865	102481588	4	RCOR1, TRAF3, AMN, CDC42BPB	1,2
q32.3	0,47	Gain	167421392	167895140	1	SPOCK	1,2
p15.1	0,01	Loss	3826471	3840780	0		
p24.2	0,07	Loss	26613719	26679147	0		1,2
q26.1	0,17	Loss	165712710	165881821	0		
		no abberations					1,2
q21.1	0,01	Loss	46365043	46378713	1	MAPK4	1,2
q34	0,91	Gain	110741933	111649459	1	ARHGEF7	1,2
q23.3	0,02	Loss	64257847	64281211	1	PLEKHG3	
q33.3	2,21	Gain	125385926	127597542	15		1,2
		no abberations					1,2
q22.11-12	1,83	Loss	34506950	36333250	9		1,2
p24.2	0,07	Loss	26613719	26679147	0		1,2
q21.1	0,00	Loss	46365043	46365939	1	MAPK4	
q14	0,18	Gain	36987323	37167451	0		1,2
p12	0,02	Loss	77088727	77105708	1	LRRTM4	
p13.32	0,03	Loss	3307020	3333041	0		
q12.1	0,00	Loss	28553357	28554420	0		
p15.2	0,09	Gain	25116956	25203433	0		1,2
q13.1-q13.2	1,29	Gain	34118096	35403781	9		1,2
		no abberations					1,2
complex pattern							1,2
q22.1-q31.33	23,75	Gain	100719050	124467674	>50		
p22-q24.3	127,47	Gain	18793861	146263538	>50		
q13.1-q13.2	0,74	Gain	34118096	34857861	6		
p11.2-q22.3	37,01	Gain	9887804	46894358	>50		
p25.3-p24.1	12,58	Loss	119769	12701540	>50		
q31.33-q36.3	34,32	Loss	124481081	158798338	>50		
q14	3,85	Loss	32680396	36528251	8		
q15.1	1,66	Loss	38336909	39998311	32		
p13.3-q25.3	78,58	Loss	18901	78599918	>50		
q11.21	1,11	Loss	18496182	19607471	13		
q13.1-q13.2	0,98	Gain	34035039	35018769	8		1,2

Supplementary Table 2: Regions of segmental telomeric uniparental disomy in 102 MDS patients

* *The column 'Reference' indicates which reference was used to exclude normal copy number variations. 1 = SNP-array analysis of individually hybridized DNA from 1015 healthy controls, 2= SNP-array analysis of DNA from pool of 10 healthy individuals hybridized simultaneously with SNP-arrays of respective MDS patients, 3= T-cells of the respective MDS patient. In addition, we compared our data with the data published in 'the database of genomic variants' as indicated in the methods section.*

** *No statistically significant correlation was seen between the occurrence of UPD in specific regions and the clinical phenotype.*

UPN	FAB	WHO	IPSS	Cytogenetics	Chr.	Cytoband	Size (Mb)	start	end	Ref Seq genes (validated)	Reference
33	RAEBt	RAEB2	int-1	46,XY, ?(17q) [7]/46,XY [3]	6	p25.3-p21.2	36,58	273303	36849394	>50	1,2,3
52	RAEBt	RAEB1	int-1	46,XY, del (9) (q22q32)	4	q13.1-q35.2	125,22	64853829	190069644	>50	1,2
68	RAEBt	RAEB2	high	46-48 XY, -5,-7, t(12;12) (p13;q22), add (21) (q22), -22x2, +r1, +r2, +r2, +mar1, +mar2, +mar3, +mar3 [cp9]/46,XY [1]	17	p13.3-p11.2	21,75	18901	21768021	>50	1,2
73	RAEB	RAEB1	int-1	47,XX,+8[7]/ 46,XX[3]	4	q12-q35.2	137,50	53370091	190870267	>50	1,2
74	RAEBt	AML	high	46,XY	4	q23-q35.2	89,79	100281453	190069644	>50	1,2
97	RA	RCMD	int-1	46,XY	7	q11.21-q36.3	95,88	61740813	157619927	>50	1,2
100	RA	RCMD	low	46,XY	4	q22.3-q35.2	94,02	97149541	191167888	>50	1,2
103	RAEB	RAEB1	int-1	46,XX	7	q21.2-q36.3	66,63	92133353	158765589	>50	1,2
105	RAEBt	RAEB2	int-2	46,XY	1	p36.33-p35.3	25,95	2095738	28045984	>50	1,2

Supplementary Table 3: Identified SNPs in the coding sequence of TET2 in MDS patients and controls

Substitution	SNP ID	Type of substitution[1]	Amino acid change	All MDS patients (n =102) n (%)	Healthy controls (n =104) n (%)	MDS patients with TET2 mutation (n =27) n (%)	Frequency in 2 cohorts (n=206) %
c.A5284G	rs2454206	ns	p.I1762V	67 (66)	59 (57)	18 (67)	61
c.T5162G	rs34402524	ns	p.L1721W	18 (18)	24 (23)	5 (19)	20
c.G5103A	rs62623390	ns	p.M1701I	3 (2.9)	1 (0.96)	2 (7.4)	1.9
c.A5333G	rs62621450	ns	p.H1778R	4 (3.9)	5 (4.8)	1 (3.7)	4.4
c.G3513A		s		1 (0.98)	2 (1.9)	0 (0)	1.5
c.C100T		ns	p.L34F	4 (3.9)	1 (0.96)	1 (3.7)	2.4
c.G1064A	rs61744960	ns	p.G355D	12 (12)	12 (12)	3 (11)	12
c.C1088T	rs17253672	ns	p.P363L	11 (11)	7(6.7)	2 (7.4)	8,7
c.C86G	rs12498609	ns	p.P29R	3 (2.9)	4 (3.8)	1 (3.7)	3.4
c.G652A	rs6843131	ns	p.V218M	4 (3.9)	4 (3.8)	1 (3.7)	3.9
c.T2599C		ns	p.Y867H	1 (0.98)	3 (2.9)	0 (0)	1.9
c.C5167T		ns	p.P1723S	1 (0.98)	3 (2.9)	0 (0)	1.9
c.A2771G	rs34485921	ns	p.H924R	0 (0)	1 (0.96)	0 (0)	0.5
c.G3117A	rs3796927	s		0 (0)	2 (1.9)	0 (0)	1.0
c.G368A		ns	p.R123H	0 (0)	1 (0.96)	0 (0)	0.5
c.G1285A		ns	p.G429R	0 (0)	1 (0.96)	0 (0)	0.5
c.A2846G		ns	p.H949R	0 (0)	1 (0.96)	0 (0)	0.5
c.A3251C		ns	p.Q1084P	0 (0)	1 (0.96)	0 (0)	0.5
c.T4140C	rs3733609	s		0 (0)	2 (0.96)	0 (0)	1.0
c.C5373T		s		0 (0)	1 (0.96)	0 (0)	0.5

[1]*s:synonymous; ns:non-synonymous*

Supplementary Table 4: Characteristics of myelopoiesis in peripheral blood used for expression analysis in granulocytes

Patient	FAB/WHO	Peripheral blood microscopy of myelopoiesis	
		Quantitative (% of total peripheral blood cells)	Qualitative
1	RARS/RCMD-RS	2% metamyelocytes, 39% band granulocytes, 1% eosinophilic granulocytes	Hyper- and hypogranulation, Pelger-Huet anomaly, abnormal nuclear chromatin
2	RA/5q- syndrome	1% promyelocytes, 9% myelocytes, 4% metamyelocytes, 8% band granulocytes, 51% segmented granulocytes	Decreased myelopoiesis, hypogranulation, abnormal nuclear chromatin, vacuolization
3	RA/RA	2% band granulocytes, 51% segmented granulocytes, 8% basophilic granulocytes, 9% eosinophilic granulocytes	Hypogranulation, abnormal nuclear chromatin, vacuolization
4	RA/RCMD	1% band granulocytes, 43% segmented granulocytes, 1% basophilic granulocytes	Hypogranulation, vacuolization
5	RA/RA	1% myelocytes, 1% metamyelocytes, 2% band granulocytes, 72% segmented granulocytes, 2% eosinophilic granulocytes	Hypogranulation, Pelger-Huet anomaly, abnormal nuclear chromatin, vacuolization
6	RAEBt/AML	14% myeloblasts, 3% myelocytes, 3% metamyelocytes, 10% band granulocytes, 11% segmented granulocytes, 1% basophilic granulocytes, 3% eosinophilic granulocytes	Hypogranulation, abnormal nuclear chromatin
7	RAEB/RAEB1	1% blasts, 1% promyelocytes, 2% myelocytes, 3% metamyelocytes, 10% band granulocytes, 54% segmented granulocytes, 3% basophilic granulocytes, 16% eosinophilic granulocytes	Decreased myelopoiesis, hypogranulation, Pelger-Huet anomaly, abnormal nuclear chromatin
8	RAEB/RAEB1	not available	not available
9	RAEB/RAEB1	49% segmented granulocytes, 1% eosinophilic granulocytes	not available

Supplementary Table 5: Allelic burden of TET2 mutations in MDS

5a: Comparison of allelic burden of TET2 mutations using pyrosequencing and chromosomal abnormalities using SNP-array analysis in 5 MDS patients.

UPN*	abnormality	location	% of affected cells**
11	missense mutation	c.5643T>G	60
	duplication	Chr. 8	80
52	missense mutation	c.2526C>A	80
	deletion	9q21.2-q31.1	70
59	missense mutation	c.1516A>T	66
	deletion	Chr. 7	74
73	missense mutation	c.3871T>C	100
	duplication	Chr. 8	73
104	frameshift mutation	c.2477delG	100
	deletion	7q31.33-q36.3	91
	deletion	5q14.3-q35.3	91
	duplication	8p22-q24.3	88

**All patients with TET2 mutations for which allelic burden was determined and which carried additional chromosomal abnormalities are included in this comparison. **Allelic burden of TET2 mutation were as described in Supplementary Table 5b. For calculation of the percentage of affected cells in large chromosomal abnormalities we used the log2 test-over-reference ratio's of chromosome X in sex-mismatched reference selections as a representative of a copy number change in all cells.*

5b: Allelic burden of TET2 mutations in MDS measured by pyrosequencing and genescan analysis

UPN	WHO	SNP-array UPD at 4q24	SNP-array del at 4q24	Mutation	pyrosequencing (% mutated allele)	genescan (% mutated allele)	estimated cells affected by TET2 mutation (%)
9	RCMD			c.3812_3813insT	N/A	44	88
				c.4571delC	49	N/A	98
11	RCMD-RS			c.5643T>G	30	N/A	60
36	RCMD-RS			c.2985_2986insA	28	N/A	56
44	RAEB2		4q24.23*	c.1147C>T	49	N/A	49
46	RCMD			c.2881G>T	48	N/A	96
				c.5688G>T	N/A	N/A	43**
				c.3854_3856delTCT	N/A	22	44
50	RCMD			c.1249C>T	30	N/A	60
52	RAEB1	4q		c.2526C>A	80	N/A	80 (homozygous)
59	RAEB1			c.1516A>T	33	N/A	66
72	RAEB1			c.1630C>T	41	N/A	82
				c.5623T>C	46	N/A	92
73	RAEB1	4q12-q35.1		c.3871T>C	100	N/A	100 (homozygous)
74	AML	4q23-q35.2		c.3508delC	100	N/A	100 (homozygous)
75	RAEB1			c.1102G>T	53	N/A	100
				c.5543C>G	52	N/A	100
77	RAEB1			c.3718_3719insT	N/A	50	100
				c.4193T>G	52	N/A	100

UPN	WHO	SNP-array UPD at 4q24 del at 4q24	Mutation	pyrosequencing (% mutated allele)	genescan (% mutated allele)	estimated cells affected by TET2 mutation (%)
79	RCMD-RS		c.5738G>A	58	N/A	100
81	RCMD		c.1720delC	43	N/A	86
83	MDS-U		c.3658delA	46	N/A	92
			c.3813C>A	N/A	N/A	77**
87	RA		c.2540_2543delAGAC	N/A	33	66
96	RCMD		c3640C>T	52	N/A	100
			c3646C>T	38	N/A	76
97	RCMD		c.4188C>G	N/A	N/A	75**
			c.5618T>C	55	N/A	100
98	RCMD		c.1665_1666insC	N/A	50	100
100	RCMD	4q22.3-q35.2	c.2045_2046insA	74	N/A	74 (homozygous)
101	RCMD		c.del5731_5748	N/A	50	100
102	RCMD		c.3782G>T	37	N/A	74
103	RAEB1		c.5455delT	N/A	35	70
			c.489_490delAC	N/A	11	22
104	AML		c.2477delG	57	N/A	100

N/A: not analyzed
**SNP-array analysis showed that this deletion was present in all cells*
*** In three cases in which pyrosequencing was inconclusive, allele burden was determined by comparison of peak-height of the mutant and wild-type allele in the original sequencing reaction*

Supplementary Table 6: Expression of TET2 mutants in bone marrow subpopulations

6a: ***Expression of TET2 mutants in bone marrow subpopulations***

UPN	Mutation	Mutation detection in reverse transcribed mRNA by PCR*			T-cells
		CD34-positive cells	CD13/33-positive cells	CD71-positive cells	
7	c5885C>T	present	N/A	present	N/A
9	c.3812_3813insT	present	present	present	N/A
	c.4571delC	present	present	present	N/A
36	c.2985_2986insA	present	present	present	N/A
72	c.1630C>T	present	present	present	absent
74	c.3508delC	present	present	present	N/A
75	c.1102G>T	present	present	N/A	absent
	c.5543C>G	present	present	N/A	absent
77	c.4193T>G	present	present	absent	absent
	c.3718_3719insT	present	present	present	absent

** To analyze the presence of mutations in different hematopoietic cell populations at the mRNA level, sequence analysis was performed on cDNA of FACS-sorted CD13/33-positive, CD34-positive and CD71-positive cells of MDS patients (n=5). N/A: not analyzed*

6b: Pyrosequencing of reverse-transcribed TET2 mRNA in bone marrow subpopulations

UPN	Mutation	Cell fraction	Pyrosequencing* (reverse transcribed mRNA)	
			Mutant allele (%)	Wild type allele (%)
9	c.4571delC	CD34	49	51
		CD13/33	45	55
		CD71	45	55
36	c.2985_2986insA	CD34	38	62
		CD13/33	N/A	N/A
		CD71	45	55
72	c.1630C>T	CD34	47	53
		CD13/33	52	48
		CD71	39	61
75	c.1102G>T	CD34	52	48
		CD13/33	52	48
		CD71	N/A	N/A
	c.5543C>G	CD34	61	39
		CD13/33	42	58
		CD71	N/A	N/A

** To determine the ratio between the expression of mutant and wild-type allele in different hematopoietic cell subpopulations, pyrosequencing was performed on cDNA of FACS-sorted CD13/33-positive, CD71-positive and CD34-positive cells of MDS patients (n=4). N/A: Not analyzed*

Supplementary Table 7: TET2 mutations in acute myeloid leukemia

UPN	Age at diagnosis	FAB	Karyotype	Molecular analysis			Type of TET2 mutation		
				Flt3**	NPM1	TET2			
AML1	71	M0	46,XY	wt	wt	wt			
AML2	66	M5	46,XY	wt	mutation	mutation	nonsense nonsense	c.2428C>T c.3813C>A	p.Q810X p.C1271X
AML3	24	unk	46,XY	wt	wt	wt			
AML4	67	M2	46,XY	wt	wt	mutation	nonsense indel	c.727C>T c.5939_5940delCA	p.Q243X p.T1980RfsX33
AML5	61	M2	46,XY	wt	mutation	wt			
AML6	68	M1/ M0	N/A (failed)	wt	wt	wt			
AML7	70	M4	46,XY	wt	wt	wt			
AML8	73	M4	46,XY	wt	mutation	mutation	nonsense indel	c.2539C>T c.383delG	p.Q847X p.S128fsX15
AML9	61	M2	47 XXY*	wt	mutation	wt			
AML10	69	M2	46,XX	wt	mutation	wt			
AML12	49	unk	N/A	mutation	wt	wt			
AML14	46	M5	46,XY	mutation	mutation	wt			
AML16	70	M5	46,XY	wt	mutation	wt			
AML17	67	M4/ M5	48,Y,add(X)(p11),?3,+8,+8, add(21)(q22)	wt	wt	wt			
AML18	70	M7	46,XY,del(13)(q14q22)[2]/46,XY,+1,der(1;7)(q10;p10)[5]/46,XY[4]	wt	wt	wt			
AML19	67	unk	46,XY, abberation chr. 17	wt	wt	wt			
AML20	76	M0/ M2	46,XY,inv(3)(q21q26)[12]	wt	wt	wt			
AML21	67	M0	46,XY,?der(5)t(1;5)(q?25;q?23)[12]/92,XXYY,?der(5)t(1;5)(q?25;q?23)x2 [3]	wt	wt	wt			
AML22	72	M1/ M2	46,XX	wt	wt	mutation	indel	c.1239delC	p.P413fsX13
AML24	41	M4	46,XY	mutation	wt	wt			

UPN	Age at diagnosis	FAB	Karyotype	Molecular analysis			Type of TET2 mutation		
				Flt3**	NPM1	TET2			
AML25	67	M4/ M5	N/A	wt	wt	muta-tion	indel	c.3353delA	p.N1118fsX17
AML26	62	M5	46,XY	muta-tion	wt	wt			
AML27	42	M5	46,XX	wt	muta-tion	wt			
AML28	53	M3	46,XX, t(15,17) (q22;q21)	N/A	wt	wt			
AML29	39	M3	46,XX, t(15,17) (q22;q21)	muta-tion	wt	wt			
AML30	46	M3	46,XX, t(4;10;15;17)	wt	wt	wt			
AML31	59	M3	46,XX, t(15;17) (q22;q21)	muta-tion	wt	wt			
AML32	38	M3	46,XX, t(15;17) (q22;q21)	muta-tion	wt	wt			
AML33	38	M3	46,XY, t(15;17) (q22;q21)	wt	wt	wt			
AML35	32	M3	46,XY, t(1;17;15)	muta-tion	wt	wt			
AML36	63	M3	t(15;17), der 3, der 8	muta-tion	wt	wt			
AML37	34	M3	46,XX, t(15;17) (q22;q21)	wt	wt	muta-tion	mis-sense	c.A2514C	p.T838P

wt = wildtype

* *Patient UPN AML9 showed this karyotypic abnormality in both bone marrow and germ-line cells.*

** *Both Flt3 ITD and Flt3 TKD mutations were analyzed. Patients UPN AML12,14,24,26, 29, 31,32 and 36 showed a Flt3 ITD. Patient UPN35 had both a Flt3 ITD and Flt3 TKD mutation.*

Supplementary Table 8: MDS patient characteristics

Characteristic	TET2 mutated n (%)	TET2 wild type n (%)
All patients	27 (26)	75 (74)
Sex ratio (Male/female) (p = 0.64)	2.4	1.7
Median age at diagnosis (p = 0.45)	67 yrs	62 yrs
FAB Classification		
RA	13 (37)	22 (63)
RARS	3 (18)	14 (82)
RAEB	8 (27)	22 (73)
RAEB-t	3 (15)	17 (85)
WHO classification		
RA	2 (20)	8 (80)
RCMD	10 (45)	12 (55)
RARS	0 (0)	6 (100)
RCMD-RS	3 (25)	9 (75)
RAEB1	7 (41)	10 (59)
RAEB2	2 (8.7)	21 (91)
Isolated 5q-	0 (0)	1 (100)
AML	2 (20)	8 (80)
MDS-U	1 (100)	0 (0)
IPSS category		
Low	12 (41)	17 (59)
Int-1	10 (29)	25 (71)
Int-2	3 (13)	21 (87)
High	2 (14)	12 (86)
Cytogenetics		
Normal	21 (32)	44 (68)
1-2 aberrations	4 (16)	21 (84)
Complex	2 (17)	10 (83)
Cytogenetics by IPSS category		
Good risk		
Intermediate	21 (31)	46 (69)
Poor risk	3 (17)	15 (83)
	3 (18)	14 (82)
Blast count in bone marrow		
<5%	17 (31)	37 (69)
5-10%	7 (35)	13 (65)
11-19%	1 (5)	19 (95)
20-30%	2 (25)	6 (75)
Cytopenias		
0/1	17 (39)	27 (61)
2/3	10 (17)	48 (83)

Supplementary Table 9: Primers

Primers for TET2 sequencing		
Primer (5'to 3')	Forward/ reverse	Location
tgtaaaacgacggccagtCAGTTTGCTATGTCTAGGTATTCCGA	Forward	Intron 2/3A
caggaaacagctatgaccAGAAGGTTCACTAACTGTGCGTTTT	Reverse	Exon 3A
tgtaaaacgacggccagtTTCAACTAGAGGGCAGCCTTG	Forward	Exon 3A
caggaaacagctatgaccTGTGCGTTTTATTCCTCCATTTT	Reverse	Exon 3A
tgtaaaacgacggccagtCAGAATAGTCGTGTGAGTCCTGAC	Forward	Exon 3A
caggaaacagctatgaccGCAATGGAAACACAATCTGGA	Reverse	Exon 3A
tgtaaaacgacggccagtGAACACACACATGGTGAACTCC	Forward	Exon 3A
caggaaacagctatgaccAATTGTGATGGTGGTGGTGG	Reverse	Exon 3A
tgtaaaacgacggccagtTCCAGGGAACCACAAAGCTAG	Forward	Exon 3A
caggaaacagctatgaccGCTTGAGGTGTTCTGACATTGG	Reverse	Exon 3A
tgtaaaacgacggccagtACATGTATGCAGCCCTTCTCC	Forward	Exon 3A
caggaaacagctatgaccGGGAATCTGCTCTTTGTTGAAA	Reverse	Exon 3A
tgtaaaacgacggccagtACCAACATCTCCAGTTCCAA	Forward	Exon 3A
caggaaacagctatgaccATGCACTTGATTTCATGGTCT	Reverse	Exon 3A
tgtaaaacgacggccagtCAAATGGGACTGGAGGAAGT	Forward	Exon 3A
caggaaacagctatgaccGTTTGCTGCTGTTCTTGCTT	Reverse	Exon 3A
tgtaaaacgacggccagtCAGAAGGACACTCAAAAGCATG	Forward	Exon 3A
caggaaacagctatgaccTTGCTGCTCTAAAGCTGGG	Reverse	Exon 3A
tgtaaaacgacggccagtGAGAATCCACCTGCAAGCTG	Forward	Exon 3A
caggaaacagctatgaccTTTCACAAGACACAAGCATCG	Reverse	Exon 3A
tgtaaaacgacggccagtGGCCACATTTTCCTAATAGATCAGTCCA	Forward	Intron 3B/3C
caggaaacagctatgaccCTGCTTTGTGTGTGAAGGCTG	Reverse	Intron 3C/4
tgtaaaacgacggccagtTTCATTTCTCAGGATGTGGTCATAG	Forward	Intron 3C/4
caggaaacagctatgaccCCAATTCTCAGGGTCAGATTTACA	Reverse	Intron 4/5
tgtaaaacgacggccagtGTTGCCCTAATTGTGATCTAAACATG	Forward	Intron 4/5
caggaaacagctatgaccAGATTGGGCTTTCCTATCAGTGG	Reverse	Intron 5/6
tgtaaaacgacggccagtGGGTTCTACTTAACTGGGTATTTTCCA	Forward	Intron 5/6
caggaaacagctatgaccAGCTTACCAAGATATGTCATATTGTTCAC	Reverse	Intron 6/7
tgtaaaacgacggccagtGGATTCAAAATGTAAGGGAATAATC	Forward	Intron 6/7
caggaaacagctatgaccGCAAGTGGTTTCAACAATTAAGAGGA	Reverse	Intron 7/8
tgtaaaacgacggccagtCCATGTCAAGATATTTGCTCTATTTTGT	Forward	Intron 7/8
caggaaacagctatgaccCAGCCATGTGGAACTGTGAGTC	Reverse	Intron 8/9
tgtaaaacgacggccagtCCACCAACACAAATCTGAATACTGA	Forward	Intron 8/9
caggaaacagctatgaccAAGTTGATGGGGGCAAAACC	Reverse	Intron 9/10
tgtaaaacgacggccagtTCTTTGCTTAATGGGTGTCGTATATC	Forward	Intron 9/10
caggaaacagctatgaccTTGGGTATTCTGATTCAAAAGCC	Reverse	Exon 10
tgtaaaacgacggccagtCCAGCCCTATGAACTTCTATTCCA	Forward	Exon 10
caggaaacagctatgaccCGGAGCTGCACTGTAGTTATGG	Reverse	Exon 10
tgtaaaacgacggccagtCAAACATGGACTATAAAAATGGTGAAC	Forward	Exon 10
caggaaacagctatgaccAAGACGAGGGAGATCCTGGTG	Reverse	Exon 10
tgtaaaacgacggccagtGCCGTGGCTCCAACTCAT	Forward	Exon 10
caggaaacagctatgaccGTGACCTTTCCCCACTGCC	Reverse	Exon 10
tgtaaaacgacggccagt	Forward	Universal sequencing primer
caggaaacagctatgacc	Reverse	Universal sequencing primer

Primers for genescan analysis of TET2		
Primer (5'to 3')	Forward/ reverse	UPN
CAAACATGGACTATAAAAATGGTGAAC	Forward	103
CACCAGGATCTCCCTCGTCTT	Reverse	103
CAAATGGGACTGGAGGAAGT	Forward	87
GTTTGCTGCTGTTCTTGCTT	Reverse	87
GGGTTCTACTTAACTGGGTATTTTCCA	Forward	9,46
GTGAACAATATGACATATCTTGGTAAGCT	Reverse	9,46
GCCGTGGCTCCAACTCAT	Forward	101
GGCAGTGGGGAAAGGTCAC	Reverse	101
GTTGCCCTAATTGTGATCTAAACATG	Forward	77
CCACTGATAGGAAAGCCCAATCT	Reverse	77
ACATGTATGCAGCCCTTCTCC	Forward	98
GGGAATCTGCTCTTTGTTGAAA	Reverse	98
tgtaaaacgacggccagt FAM-labeled	Forward	All
caggaaacagctatgacc FAM-labeled	Reverse	All

Primers for pyrosequencing of TET2				
Primers for amplification	Forward/ reverse	Primers for sequencing	UPN	Mutation
GGTTCCAGCAGCAATTTG	forward	CCTGGTGGCAGCTCT	75	c.1102G>T
AGCGCTGCTCCGGTTCATAGATTGAACACTGAGCTTTGCTTGAAGTA	reverse			
GACGGGACACCGCTGATCGTTTATGGCAGCTCTGAACGGTATTT	forward	TAGTGAACACTGAGCTTT	44	c.1147C>T
TGTGGTAGTGGCAGAAAAGGAAT	reverse			
AATTGCTTCTTTCTCCCCCTCC	forward	TCCTCTTCCACAGGTT	50	c.1249C>T
AGCGCTGCTCCGGTTCATAGATTCCACCATTCAGAGTGCTTTTTC	reverse			
GCAGGGACAATGACTGTTCCA	forward	CCATTGTGTTCTGAGAAA	59	c.1516A>T
AGCGCTGCTCCGGTTCATAGATTGGTGGGTTATGCTTGAGGTGTT	reverse			
GACGGGACACCGCTGATCGTTTAGAAACAAAGAGCAAGAGATTCTGA	forward	CGTGTTTGCTCCTTGTC and GGTTTCAGATAGTGCTGTG	72, 98	c.1630C>T and c.1665_1666insC
GGCCTTCAATTCAATCCATC	reverse			
GACGGGACACCGCTGATCGTTTAATTGAAGGCCCCTCGTTT	forward	TTTAGATGGGATTCCG	81	c.1720delC
AGTGATGCCTCATTACGTTTTAGA	reverse			
GACGGGACACCGCTGATCGTTTATCAACCCAATCTCTCCAATCAAA	forward	CCCAGGCATGTTGGAA	86	c.1821delA
TGTGTTGTTTTCTGGGTGTAAGC	reverse			
GACGGGACACCGCTGATCGTTTATGCAGTCACTGTGTGGCACTA	forward	TCTGCTCTTTGTTGAAAA	100	c.2045_2046insA
GCTGTTTCAACACTGGGGACAT	reverse			
GACGGGACACCGCTGATCGTTTATTCCCCTTATAGTCAGACCATGAA	forward	GAAACCTGTATTTTGCAT and GTGTAGTCTGTTCTTTATTC	52, 104	c.2526C>A and c.2477delG
GGGTCTTGTTTCCTGCAAAAAGT	reverse			
AGGTGGCATCTCTTACAGAAGCA	forward	TGGCATCTCTTACAGAAG	46	c.2881G>T
AGCGCTGCTCCGGTTCATAGATTAAGACTCAGTTTGGGGTTGCT	reverse			
CAGATGCACAGGCCAATTAA	forward	ATGCCTGTATGCACAC	36	c.2985_2986insA
AGCGCTGCTCCGGTTCATAGATTGGTGGATTCTCTTGCTTAGTTACC	reverse			

GTGTTTCTGTGGGTTTCTTTAAGG	forward	GGTTTCTTTAAGGTTTGG	74	c.3508delC
AGCGCTGCTCCGGTTCATAGATTGAGAACTTTTGCCTTCTTTACCAG	reverse			
GGGTGGTTCGCAGAAGCA	forward	GAGCGAGCTGGCCAC and AGAAGCTACTGTGTTTGG	83, 96	c.3658delA and c3640C>T and c3646C>T
AGCGCTGCTCCGGTTCATAGATTCTTCCCACACCAGGATGAGAATC	reverse			
GACGGGACACCGCTGATCGTTTACATCCTGGTGTGGGAAGGAATC	forward	AGTAGAGTTTGTCAGCCA and CATTCAAGGCACACC	77, 102	c.3718_3719insT and c.3782G>T
TTCCTATCAGTGGCCGCAAAG	reverse			
TTTGGTCTTTTGATTTTTCAGG	forward	TTGATTTTTCAGGAGAACT and CCTTCTCTTTTGGTTGTT	9, 73, 83	c.3812_3813insT and c.3871T>C and c.3813C>A
AGCGCTGCTCCGGTTCATAGATTCTTAAACTTCCTTGGGATCTTGC	reverse			
ACGTTTTCTTTGGGACCTGTAGT	forward	TTACTTCCCTACCAGGTAT	77, 97	c.4193T>G and c.4188C>G
AGCGCTGCTCCGGTTCATAGATTCCTCCAAATTCTCGATTGTC	reverse			
GACGGGACACCGCTGATCGTTTACAGCTTCACGTTCTGCCTTTATA	forward	CCAAACTCATCCACG	9	c.4571delC
GGCACCACTCCGTTTTTTCT	reverse			
GACGGGACACCGCTGATCGTTTATCCCTGGAGAACAGCTCAAATA	forward	ACGTGATGGGGCTGA	86	c.4863delA
GGCTTGCGTTTTCAGTTTGTTT	reverse			
GACGGGACACCGCTGATCGTTTACAAAGATGCTTCCAGCTCTTAACC	forward	GCTCTGCTCGCTGTCT and TGACCATTAGCATCACTT	75, 103	c.5543C>G and c.5455delT
CGCTTTGCACACTCAATGAGA	reverse			
CGAGCAGAGCTTTCTGGATCCT	forward	GCAAAGCGTGAGCTG and CTCATGGGTCAATTCTC	11, 72, 97	c.5643T>G and c.5623T>C and c.5618T>C
AGCGCTGCTCCGGTTCATAGATTAGATCCTGGTGGGGTGATTCC	reverse			
AGAATCCCAATAGGAATCACCC	forward	AATAGGAATCACCCCAC and TGAATGAGCCAAAACA	46, 79	c.5688G>T and c.5738G>A
AGCGCTGCTCCGGTTCATAGATTTCTGAGGCACATAGTCTGG	reverse			
GACGGGACACCGCTGATCGTTTAAGTGAAACGGGAGCCTGCT	forward	TGATGAAACGCAGGTA	7	c.5885C>T
GGTCCTTTCGGCAAGAGACTT	reverse			

Primers for confirmation TET2 splice variants		
Primer (5' to 3')	Forward/ Reverse	Location
tgtaaaacgacggccagtACAGAAGGTGGGCCGGGGCGG	Forward	1A
tgtaaaacgacggccagtAGAACTCGGTCAATTTCCCAGTT	Forward	1A
tgtaaaacgacggccagtGGGAGAAACAGAACTCGGTC	Forward	1A
tgtaaaacgacggccagtGTCGGGTCTTTAAAAATACAGG	Forward	1A
TAAAGGGAGATAGAGACGCG	Forward	1A
TATTGATGCGGAGGCTAGGC	Forward	1B
CAAACATTCAGCAGCACACC	Forward	2
CCTTACTGCTCTTCCTGGATC	Reverse	2
CCTGGATCATGTCCTATTGGCT	Reverse	2
GACACCAACCAAAAGAACAGC	Forward	3A
TGACTAGACAAACCACTGCTGC	Forward	3A
GTGCGTTTTATTCCTCCATTTT	Reverse	3A
caggaaacagctatgaccGGAGCTTTGTAGCCAGAGGT	Reverse	3A
caggaaacagctatgaccTGTGCGTTTTATTCCTCCATTTT	Reverse	3A
GATAAACGCCATGTGTCTCAGTACA	Reverse	3B
CATGATTTCTCTAATAGCTGCCACA	Reverse	3C
CAATAGGACATCCCTGAGAACTT	Reverse	4

Primers and probe for isoform-specific quantitative TET2 PCR			
Primer/probe (5'to 3')	Forward/ reverse	Location	Isoform specificity
AATTTATTGGATACACCTGTCAAGACTC	Forward	Exon 3A	1,2,3
GATAAACGCCATGTGTCTCAGTACA	Reverse	Exon 3B	2
ACCTGCTCCTAGATGGGTATAAAAAG	Reverse	Exon 3C	1
TTTACCCTTCTGTCCAAACCTACAC	Reverse	Exon 3A/4	3
TATGATTTCCCATCTTGC	Probe	Exon 3A	1,2,3

Chapter 4

TET2 mutations in childhood leukemia

Leukemia 2011;25 (1): 189-192

SMC Langemeijer, JH Jansen, J Hooijer, P van Hoogen, E Stevens-Linders, M Massop, E Waanders, SV van Reijmersdal, MJPL Stevens-Kroef, CM Zwaan, M van den Heuvel, E Sonneveld, PM Hoogerbrugge, A Geurts van Kessel, RP Kuiper

Abstract

Aberrations of the Ten-Eleven-Translocation 2 (*TET2*) gene have been implicated in a variety of hematopoietic malignancies in adults, but not in childhood malignancies. We studied a cohort of childhood acute myeloid leukemia (AML) and precursor B-cell acute lymphoblastic leukemia (B-cell ALL) for the presence of mutations in *TET2*. In total 104 childhood AML and 47 childhood precursor B-cell ALL cases were included. *TET2* mutations were detected in 4 of the 104 AML samples tested (3,8%). No *TET2* mutations were found in the precursor B-cell ALL cohort. The observation of the presence of *TET2* mutations in childhood AML may be instrumental for the design of novel targeted therapies.

The Ten-Eleven-Translocation 2 (*TET2)* gene has recently been implicated in a variety of hematopoietic malignancies in adults. Mutations and deletions were detected in myelodysplastic syndromes (MDS), acute myeloid leukemias (AML) and other myeloid malignancies[1-5].

So far, the presence of *TET2* mutations has not been reported in childhood leukemia. Precursor B-cell ALL and AML represent the most frequently occurring hematopoietic malignancies in children. During the past decades, the survival rates of children with these malignancies have improved considerably, but a substantial subset of the patients still suffers from relapses and treatment-related mortality. Genetic abnormalities may provide insights into the mechanisms underlying the etiology of leukemia and the development of therapy resistance. In addition, they may provide novel clues for the development of targeted therapies. *TET2* mutations are found in 8-19% of adult AML[2, 5]. Thus far, the incidence of TET2 mutations in adult ALL has not been reported. *TET2* expression is high in granulocytes where it is increased during myeloid differentiation, and in normal B-cells as compared to other hematopoietic and non-hematopoietic cells[2]. Here, we set out to assess a cohort of childhood AMLs and precursor B-cell ALLs for the presence of mutations in *TET2*.

In total 104 childhood AML and 47 childhood precursor B-cell ALL cases were included in this study. All patient material was obtained with informed consent through the Dutch Childhood Oncology Group (DCOG). Patient characteristics are listed in **Supplemental Table 1**. DNA was isolated from peripheral blood or bone marrow cells at diagnosis. Sequence analyses were carried out as reported before using primers covering all exons of the *TET2* gene, including its intron-exon boundaries[2]. All sequence analyses were performed bi-directionally and all sequence variations were confirmed independently. In order to establish whether the observed mutations were acquired, DNA from remission material was analyzed whenever available. All sequence variations were compared to those published in the literature and listed in public databases.

Novel *TET2* mutations were detected in 4 of the 104 AML samples tested (3.8%) **Figure 1a, Table 1**). In addition, 16 SNPs were found, some of which have not been reported before (**Figure 1a**). One of the patients carrying a *TET2* mutation, a 3-month old girl (UPN AML51), suffered from AML-M0. A conversion of cytosine to guanine at position 1526 of the *TET2* gene led to the formation of a premature stop codon. A second patient, a 17-year-old boy (UPN AML60), suffered from AML-M2. In his case, the *TET2* mutation (c.3001A>T) gave rise to a premature stop codon as well. In both cases the mutations were not detected in DNA obtained during remission. Both premature stop codons were located in the N-terminal moiety of the protein, upstream of the two conserved domains BOX1 and BOX2 (**Figure 1a**). The two other patients with *TET2* mutations suffered from AML-M4. In one of them, a 14-year-old girl (UPN AML07), the mutation (c.1379C>T) led to a conversion of a serine to a phenylalanine, again upstream of the two conserved domains in the protein. Unfortunately, remission DNA from this patient was not available for analysis, but literature and database searches revealed that this mutation has been reported before and was shown to be acquired[5]. In the fourth patient, the diagnosis sample showed a homozygous mutation of one base-pair upstream of the GT-splice-donor site of intron 5. This mutation, an insertion of a thymine, was absent in remission material (**Figure 2a**). In addition to the mutation, we detected two SNPs which were homozygous in the diagnosis sample, but heterozygous in the remission sample (**Figure 2a**), indicative for the presence of either a deletion or a region of acquired

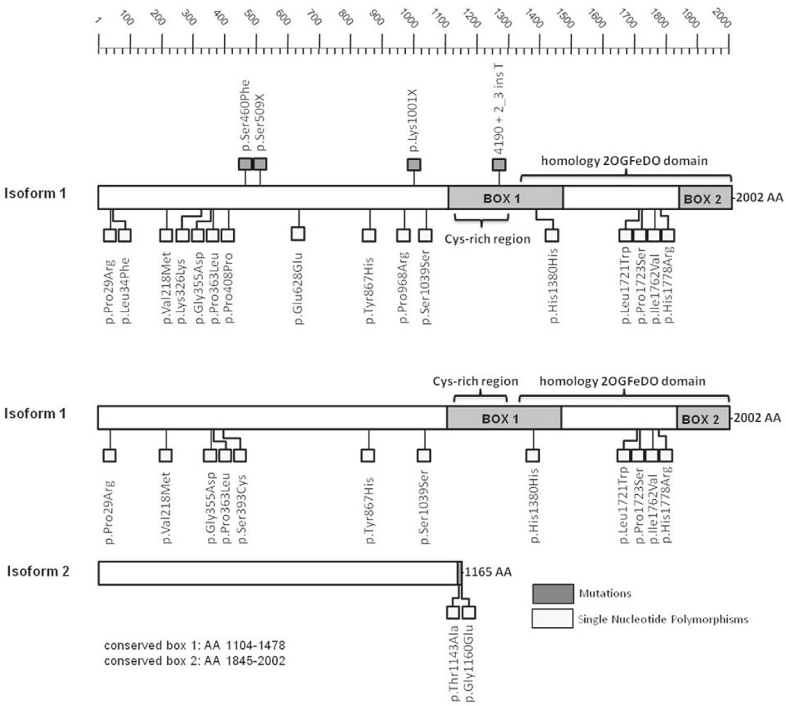


Figure 1: Schematic representation of the TET2 protein showing the localization of the conserved domains BOX1 and BOX2, and the mutations and SNPs found in pediatric AML (a) and precursor B-cell ALL (b)

Table 1: Characteristics of the four TET2-mutated patients

UPN	AML51*	AML60	AML07**	AML19#
TET2 mutation cDNA	c.1526C>G	c.3001A>T	c.1379C>T	4190 +2_3 ins T
TET2 mutation protein	p.Ser509X	p.Lys1001X	p.Ser460Phe	unknown
Age at diagnosis (years)	0	17	14	11
Sex	female	male	female	female
Blasts bone marrow (%)	85	48	85	87
Blasts peripheral blood (%)	95	46	86	83
Subtype	M0	M2	M4	M4
CNS involvement	yes	no	no	no
Cytogenetics	47,XX,+19 [8]/ 46,XX [2] FISH: t(7;12)	unknown	46,XX, del(8) (p11), del(16)(p11), add(17)(p13) [10]	46,XX [20]
Co-occurence of other mutations	no*	unknown	no**	*NPM1* and *FLT3 ITD**
TET2 mutation previously described (references)	yes (3, 4)	no	yes (5)	no

** mutation screening negative for CEBPalpha, NPM1, WT1, c-KIT, N-RAS, K-RAS, FLT3 ITD, FLT3 TKD, PTPN11*

*** mutation screening negative for NPM1 and MLL-PTD*

 # mutation screening positive for NPM1 (exon 12 CCTG, type D) and FLT3 ITD. Negative for CEBPalpha, WT1, c-KIT, N-RAS, K-RAS, FLT3 TKD, PTPN11, MLL-PTD

uniparental disomy (aUPD) affecting the *TET2* gene. Subsequent SNP array-based genomic profiling of the respective DNA samples indeed confirmed the presence of an aUPD of the long arm of chromosome 4 harboring the *TET2* gene (**Figure 2b**).

No *TET2* mutations were found in the precursor B-cell ALL cohort (**Figure 1b**). The locations of 14 *TET2* SNPs are depicted in Figure 1b. In two patients we detected sequence variations leading to amino-acid substitutions in the isoform 2 of TET2. Remission material of these patients was not available for analysis, but considering the fact that these variations only affect the short TET2 isoform lacking the two conserved domains BOX1 and BOX2, we consider that the functional consequences of these variations may be limited. One of the variations (p.Gly1160Glu) was also encountered in a cohort of adult AML patients (unpublished results), and was found to be present in remission material. Therefore, we concluded that this variation represented a SNP.

Compared to the incidence of *TET2* mutations in adult AML, its incidence in childhood AML seems to be lower (8-19% versus 3.8%). In adult AML *TET2* mutations appear to correlate with a poor prognosis ([5], unpublished results). As yet, however, our childhood AML cohort is too small to draw any firm conclusions on its prognostic implications.

In our precursor B-cell ALL cohort no *TET2* mutations were found. Previously, we and others performed SNP array-based analyses on precursor B-cell ALLs[6, 7] and identified recurrent deletions, duplications and aUPDs affecting various regions on the genome, but the 4q24 region encompassing the *TET2* gene was not affected.

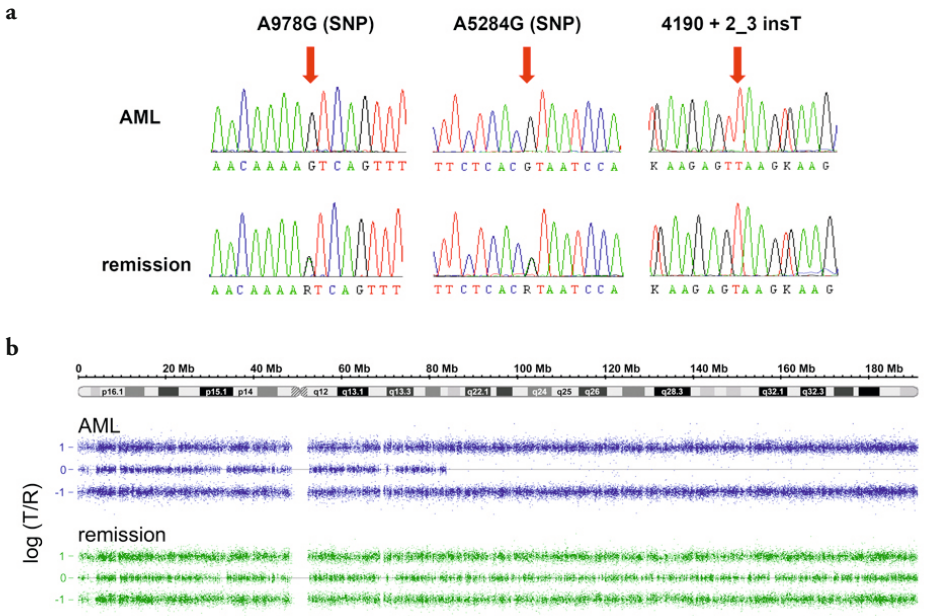


Figure 2: A homozygous TET2 splice site mutation in a child with AML.
(a) Sequence analysis in leukemic and remission samples of patient UPN AML19 revealed two heterozygous SNPs with acquired homozygosity in the diagnosis sample and a homozygous mutation in the splice donor site of exon 5 (c.4190+2_3insT). (b) Genotyping plot showing the allelic difference of all SNPs on chromosome 4, with acquired uniparental disomy of a segment of chromosome 4q. No deletions were detected in this region.

The mechanism by which *TET2* mutations contribute to the etiology of myeloid malignancies is, as yet, poorly understood. Tahiliani et al. have implicated that the *TET2* homolog *TET1* may be involved in epigenetic gene regulation[8]. Specifically, they found that TET1 plays a role in the transition of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC). Very recently TET2 was reported to possess similar enzymatic activity[9]. Considering the co-occurrence of *TET2* mutations with other mutations such as *FLT3 ITD, TET2* mutations are probably type II mutations. In conclusion, we have detected novel *TET2* mutations in 3.8% of pediatric AML patients. This observation may be instrumental for the design of novel targeted therapies in pediatric acute myeloid leukemia.

Reference List

1. Delhommeau,F. et al. Mutation in TET2 in myeloid cancers. N. Engl. J. Med. 360, 2289-2301 (2009).

2. Langemeijer,S.M. et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat. Genet. 41, 838-842 (2009).

3. Tefferi,A. et al. TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23, 905-911 (2009).

4. Jankowska,A.M. et al. Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. Blood 113, 6403-6410 (2009).

5. bdel-Wahab,O. et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 114, 144-147 (2009).

6. Mullighan,C.G. et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature 446, 758-764 (2007).

7. Kuiper,R.P. et al. High-resolution genomic profiling of childhood ALL reveals novel recurrent genetic lesions affecting pathways involved in lymphocyte differentiation and cell cycle progression. Leukemia 21, 1258-1266 (2007).

8. Tahiliani,M. et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930-935 (2009).

9. Ito,S. et al. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 466, 1129-1133 (2010).

Supplementary information

Supplementary Table 1: Patient characteristics

	AML	ALL
Number of patients	104	47
Time of diagnosis	1982 to 2009	1992 to 1996
Age at diagnosis, median (range)	6 (0-17)	4 (0-14)
Sex (male / female / unknown)	69 /34 / 1	29 / 18
Blasts bone marrow, median (range)	80 (15-97)	90 (64-99)
Blasts peripheral blood, median (range)	66 (2-95)	37 (0-95)
Subtype AML		
M0	9	
M1	9	
M2	22	
M3	10	
M4	23	
M5	24	
M6	1	
M7	3	
RAEBt	1	
unknown	2	
Subtype ALL		
pro-B-ALL		1
pre-B-ALL		13
c-ALL		33
CNS involvement (Y/N/unknown)	9 / 61 / 34	0 / 34 / 13
Cytogenetics		
normal	19	0
high hyperdiploidy (>50)	1	13
triple trisomy (4,10,17)	0	7
hypodiploidy (<46)	6	5
t(8;21)	14	0
t(15;17)	8	0
inv(16)/t(16;16)	6	0
t(11q23)/MLL	19	0
t(9;22)	0	2
t(1;19)	0	1
t(12;21)	0	0
Relapse	37	24

Chapter 5

Somatic mutations of the histone methyltransferase gene *EZH2* in myelodysplastic syndromes

Nature Genetics 2010; 42(8): 665-667

G Nikoloski*, SMC Langemeijer*, RP Kuiper, R Knops, M Massop, ERLTM Tönnissen, A van der Heijden, TN Scheele, P Vandenberghe, T de Witte, BA van der Reijden & JH Jansen

*contributed equally

Abstract

In myelodysplastic syndromes (MDS), deletions of chromosome 7 or 7q are common and correlate with a poor prognosis. The relevant genes on chromosome 7 are unknown. We report here that *EZH2*, located at 7q36.1 is frequently targeted in MDS. Analysis of *EZH2* deletions, missense and frameshift mutations strongly suggests that *EZH2* is a tumor suppressor. As *EZH2* functions as a histone methyltransferase, abnormal histone modification may contribute to epigenetic deregulation in MDS.

Myelodysplastic syndromes comprise a heterogeneous group of acquired clonal hematopoietic disorders characterized by dysplasia of the myeloid, erythroid and/or megakaryocytic lineages. MDS is one of the most frequent hematopoietic malignancies, particularly in the elderly[1]. In the majority of affected individuals, a cure for the disease cannot be achieved and most die from severe cytopenias. Identification of the underlying genetic aberrations may promote proper classification and prognostication of disease and, eventually, the development of targeted therapy.

Conventional cytogenetic techniques detect large chromosomal aberrations in 40-60% of individuals with MDS, of which deletions of chromosome 5 or 5q (15%) and 7 or 7q (10%) are the most frequent[2]. Several genes have been implicated on chromosome 5, but the relevant genes on chromosome 7 have remained elusive.

Previously, we applied single nucleotide polymorphism (SNP) arrays on a cohort of 102 subjects to identify genomic aberrations in MDS. The complete overview of SNP array lesions has been published[3]. Using DNA-sequencing, we and others identified the perturbation of the *TET2* gene located at chromosome 4q24, in 26% of the individuals with MDS[3, 4]. Here, the same cohort was used to identify affected genes on chromosome 7. SNP array analysis revealed chromosome 7 deletions in 14 out of 102 subjects and loss of heterozygosity due to uniparental disomy (UPD) in two subjects (**Figure 1a**). In subject 87, we found a monoallelic microdeletion of 130 kb at 7q36.1. This region was shared by 13 out of 14 subjects with deletions, as well as by the two subjects with UPD and encompassed two genes, *CUL1* and *EZH2* (**Figure 1a** and **Supplementary Figure 1**). Genomic sequence analysis of subject 87 covering the coding region and splice sites of the remaining copy of these genes showed no aberrations in *CUL1*. However, a frameshift mutation in exon 7 (703delGinsAA, nomenclature as described[5]) introducing a premature stop codon was found in *EZH2* (**Table 1, Supplementary Figure 2** and **Supplementary Figure 3**). The presence of this mutation implied that no intact copy of *EZH2* was left in the malignant bone marrow cells of this individual. Because large segmental regions of UPD may harbor acquired homozygous mutations, we sequenced *EZH2* in the bone marrow cells of the two subjects who showed UPD at 7q (**Figure 1a**, subjects 97 and 103). *EZH2* mutations were not detected in subject 103, but a homozygous missense mutation was present in subject 97 (**Table 1**).

To assess the prevalence of *EZH2* mutations in individuals with MDS, we sequenced the gene in all 102 subjects from the initial cohort and 24 additional individuals with MDS (**Supplementary Table 1**). *EZH2* missense, donor-splice-site and frameshift mutations predicting truncated proteins were observed in 8 out of 126 subjects (6%, **Table 1** and **Figure 1b**). In three individuals we could show that mutations were biallelic. In case 87, one allele was deleted whereas the other contained a point mutation. In subject 97, a point mutation was found that was homozygous due to UPD, and in subject 96 two different point mutations were found that resided on two different alleles (**Supplementary Figure 4**). To investigate whether these mutations were somatically acquired, we analyzed DNA of non-neoplastic T cells from the same individuals. Polyclonal T cell cultures were generated from viably frozen cells of subjects 10, 40 and 73 (**Supplementary Methods**). None of the four mutations detected in the malignant cells were found in these T cells, confirming these were acquired mutations (**Table 1**). For two additional subjects who carried missense mutations

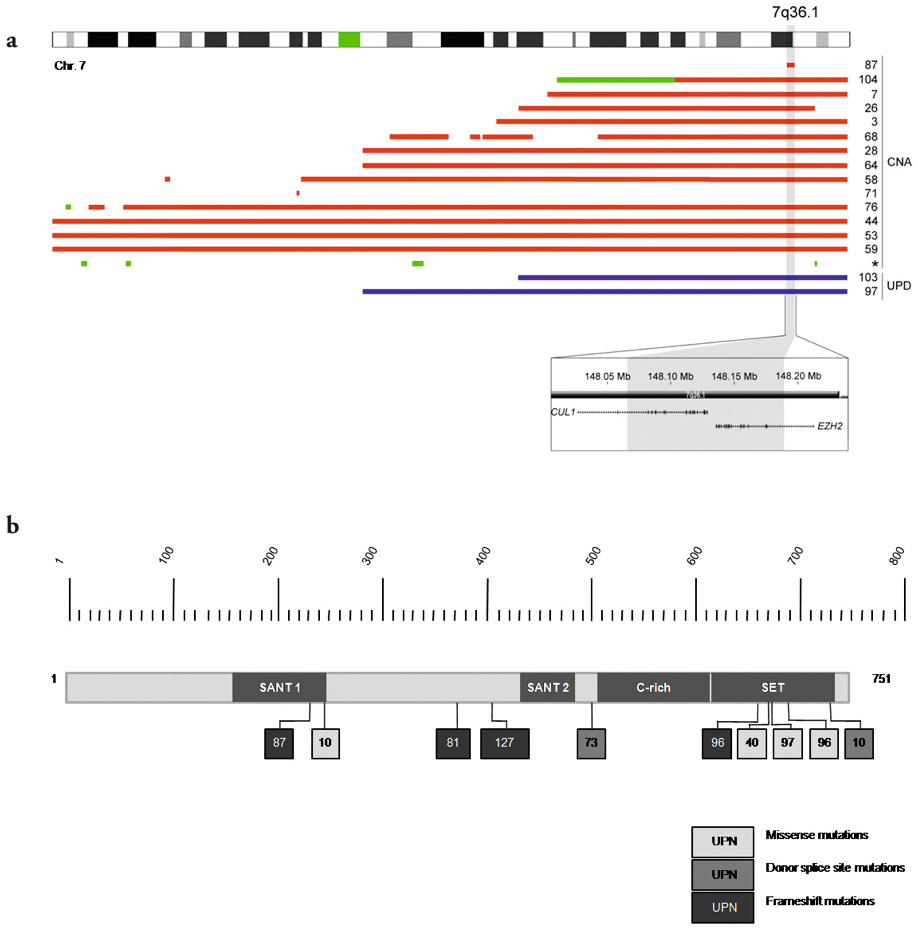


Figure 1: EZH2 is recurrently affected in individuals with MDS.
(a) Genomic aberrations of chromosome 7 detected in 102 individuals with MDS by high-resolution SNP arrays. Numbers on the right are unique patient numbers. Two subjects carried large areas of UPD (blue bars), fourteen subjects carried deletions (red bars) and six showed duplications (green bars, asterisks represent four different subjects). Subject 87 showed a 130-kb microdeletion at 7q36.1, which contains CUL1 and EZH2. CNA, Copy Number Alterations; UPD, Uniparental disomy. (b) Protein localization of ten different EZH2 point mutations found in 8 out of 126 subjects with MDS. The ruler indicates the number of amino acids. The catalytic SET domain, a C-rich domain and the two SANT domains are indicated. Numbers in the boxes indicate unique patient numbers (UPN)

Table 1: Aberrations of EZH2 at chromosome 7q36.1 were detected in 29 bone marrow samples of 126 individuals with MDS.
*Genomic sequencing of the coding region and the splice donor and acceptor sites revealed missense mutations, donor-splice-site mutations, deletions and insertions, predicting amino acid substitutions and truncations. Sequences were compared to the reference genomic sequence NT_007914.15 and the deduced protein sequences to reference NP_004447.2. In subjects 44 and 104, the 7q deletion that was identified on SNP array was missed by karyotype analysis. In subject 43, the 7q deletion that was revealed by karyotype analysis was not detected by SNP array analysis. To assess whether mutations were acquired, T cells from the same individuals were analyzed or allelic discrimination assays were applied to show that the mutations were absent in a cohort of 250 unaffected donors (Supplementary Methods, Supplementary Table 2 and Supplementary Figure 5). Detected SNPs are summarized in Supplementary Table 3. UPN, Unique Patient Number; FAB, French-American-British classification; WHO, World Health Organization classification; IPSS, International Prognostic Scoring System; N/A, Not Analyzed, mutation is not present in the NCBI SNP databases; *mutation was absent in a cohort of 250 healthy donors.*

Table 1 Characteristics of MDS patients showing 7q36.1/EZH2 aberrations

	Classification				SNP array		Point mutations in EZH2			
UPN	FAB	WHO	IPSS	Karyotype	UPD at 7q36.1	Del at 7q36.1	DNA variation	Protein variation	Homo-, hetero- or Hemizygous	Acquired or Inherited
3	RAEB-t	RAEB-2	High	complex with chr 7 lesions	No	7q21.13-q36.3				
7	RAEB-t	RAEB-2	Int-2	complex with chr 7 lesions	No	7q31.1 -q36.3				
10	RAEB-t	RAEB-2	Int-2	normal	No	No	c.745G>A	p.E249K	Heterozygous	Acquired
							c.2195+1G>A	p.Y733LfsX6	Heterozygous	Acquired
26	RA/RAEB	RA/RAEB-1	Int-2	del(7)(q22), del(20)(q11)	No	7q21.3 -q36.2				
28	RAEB	RAEB-1	Int-2	+1, der (1;7)(q10;p10)	No	7q11.21-q36.3				
40	RAEB	RAEB-2	High	t(3;16)(q26;q2?3)	No	No	c.2020T>G	p.L674V	Heterozygous	Acquired
43	MDS-U	MDS-U	Int-2	del (5)(q13q33), -7	No	No				
44	RAEB	RAEB-2	Int-2	normal	No	7p22.3 -q36.3				
53	RAEB	RAEB-1	Int-2	complex with chr 7 lesions	No	7p22.3 -q36.3				
58	RAEB	RAEB-2	High	complex with chr 7 lesions	No	7p12.3 -q36.3				
59	RAEB	RAEB-1	Int-2	-7	No	7p22.3 -q36.3				
64	RAEB	RAEB-2	High	complex with chr 7 lesions	No	7q11.21-q36.3				
68	RAEB-t	RAEB-2	High	complex with chr 7 lesions	No	7q22.1 -q36.3				
73	RAEB	RAEB-1	Int-1	+8	No	No	c.1505+1G>T	unknown	Heterozygous	Acquired
76	RAEB-t	AML	High	complex with chr 7 lesions	No	7p21.2 -q36.3				
81	RA	RCMD	Int-1	normal	No	No	c.1119-1120insC	p.T374HfsX3	Heterozygous	N/A
87	RA	RA	Low	normal	No	7q36.1	c.703delGinsAA	p.G235KfsX11	Hemizygous	N/A
96	RA	RCMD	Low	normal	No	No	c.1983delA	p.V662CfsX13	Heterozygous	N/A
							c.2068C>T	p.R690C	Heterozygous	N/A*
97	RA	RCMD	Int-1	normal	7q11.21-q36.3	No	c.2025C>A	p.N675K	Homozygous	N/A*
103	RAEB	RAEB-1	Int-1	normal	7q21.2 -q36.3	No				
104	RAEB-t	AML	High	complex	No	7q31.33-q36.3				
106	RA	RA	Int-2	-7	N/A	N/A				
108	RAEB	RAEB-1	Int-1	complex with chr 7 lesions	N/A	N/A				
112	RARS	RCMD-RS	Int-1	complex with chr 7 lesions	N/A	N/A				
113	RAEB	RAEB-1	Int-2	-7	N/A	N/A				
114	RA	RCMD	Int-2	der(7)t(1;7)(p10;q10)	N/A	N/A				
119	RAEB	RAEB-1	Int-2	del(5)(q15q33),	N/A	N/A				
				der(7)del(7)(p11)add(7)(q3?3)						
121	RAEB	RAEB-1	Int-2	complex with chr 7 lesions	N/A	N/A				
126	RA	RCMD	Int-2	complex with chr 7 lesions	N/A	N/A				
127	RA	RA	Low	normal	N/A	N/A	c.1212-1216delGAAGA	p.K405RfsX2	Heterozygous	N/A

(subjects 96 and 97), we designed allelic discrimination assays (PCR using allele-specific probes and high-resolution melting analysis, HRM) and tested a cohort of 250 unaffected individuals (controls) (**Supplementary Methods** and **Supplementary Table 2**). None of these mutations were found in any of the unaffected subjects (**Table 1** and **Supplementary Figure 5**). In three individuals, we examined whether the mutant mRNA was expressed (**Supplementary Figure 6 and Supplementary Methods**). In subject 73 (who harbored a heterozygous splice-donor-site mutation in intron 12), only wild-type mRNA was detected. No aberrant splice variants could be detected using primers from exon 11 to exon 13, or when exon 11 to 20 primers were used (**Supplementary Methods**). In subjects 10 and 127, mutant mRNA was detected, but pyrosequencing and Genescan analysis showed that the mutant sequences were expressed at lower levels than wild-type sequences, suggesting a decreased stability of the mutant transcript. Although we could readily detect EZH2 protein in cell lines and in primary T cells, no EZH2 protein expression could be measured in total bone marrow of individuals with MDS, irrespective of the presence or absence of *EZH2* mutations. Therefore, more detailed expression studies in bone marrow subfractions of MDS patients are warranted. In addition to the eight subjects with *EZH2* point mutations, the *EZH2* locus at 7q36.1 was entirely deleted at one allele in 22 subjects by 7 or 7q (micro)deletions. Collectively, deletions and point mutations of *EZH2* were present in 23% of the affected subjects. In 5 out of 8 subjects with an EZH2 mutation, a TET2 mutation was present (ref. 3 and **Supplementary Table 1)**. Overall, in 40% of the subjects showing 7q36.1 aberrations or *EZH2* mutations, *TET2* was affected as well. This is notable, as both EZH2 and the Tet family of proteins have been implicated in epigenetic regulation of gene transcription[6-10].

The cohort harboring *EZH2* aberrations was too small to detect a clear association with a specific French-American-British (FAB), International Prognostic Scoring System (IPSS) or World Health Organization (WHO) classification of MDS (**Table 1**). Subjects carrying 7 or 7q deletions showed a significantly worse survival compared to patients without any *EZH2* abnormalities ($p<0.001$, **Supplementary Figure 7**). The adverse effect of chromosome 7 and 7q deletions on the prognosis in MDS is well known[1]. We show here that *EZH2* point mutations result in a similar adverse tendency in overall survival ($p=0.076$ when measured from the date of diagnosis and $p=0.026$ when measured from the time of bone marrow sampling), but a larger cohort from a prospective clinical trial should be analyzed to assess this properly.

EZH2 encodes the histone methyltransferase that constitutes the catalytic unit of the Polycomb Repressive Complex 2 (PRC2)[7-10]. This complex initiates dimethylation and trimethylation of lysine 27 of histone H3 (H3K27), an epigenetic modification associated with gene silencing. All the mutations detected here disrupt highly conserved amino acids (**Supplementary Figure 8**). Notably, the SET domain, essential for the methyltransferase activity of EZH2[8-10], was either altered or truncated in at least one allele in all affected subjects (**Figure 1b** and **Supplementary Figure 8**). We conclude that *EZH2* is targeted by various types of deletions and mutations in MDS, probably leading to loss-of-function of the gene. Recently, a recurring mono-allelic *EZH2* point mutation in the SET domain (Tyr641; Tyr646 in NM_004456.3) leading to loss-of-function has been described in lymphomas[11]. Together with our data, this suggests that EZH2 may acts as a tumor suppressor. This is in line with recent investigations showing that Drosophila E(z) as well as other PRC1 and

PRC2 components may exhibit tumor suppressor activity by silencing the mitogenic JAK-STAT[12] and Notch[13] signaling pathways. In contrast, in various malignancies, overexpression of *EZH2* has been reported[14], suggesting that EZH2 might also act as an oncogene. This indicates that deregulation of *EZH2* in cancer may not be generalized into one simple mechanism. We postulate that changes in epigenetic modifications caused by defective EZH2 may result in tumor promotion by different mechanisms depending on the cellular context and the oncogenic pathways that are activated.

Reference List

1. Brunning,R.D. Myelodysplastic syndromes in WHO classification of Tumours of Haematopoietic and lymphoid tissues 87-104 2008).

2. Haase,D. et al. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. Blood 110, 4385-4395 (2007).

3. Langemeijer,S.M. et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat. Genet. 41, 838-842 (2009).

4. Delhommeau,F. et al. Mutation in TET2 in myeloid cancers. N. Engl. J. Med. 360, 2289-2301 (2009).

5. den Dunnen,J.T. & Antonarakis,S.E. Nomenclature for the description of human sequence variations. Hum. Genet. 109, 121-124 (2001).

6. Tahiliani,M. et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930-935 (2009).

7. Cao,R. et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298, 1039-1043 (2002).

8. Kuzmichev,A., Nishioka,K., Erdjument-Bromage,H., Tempst,P., & Reinberg,D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. 16, 2893-2905 (2002).

9. Czermin,B. et al. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111, 185-196 (2002).

10. Muller,J. et al. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111, 197-208 (2002).

11. Morin,R.D. et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat. Genet. 42, 181-185 (2010).

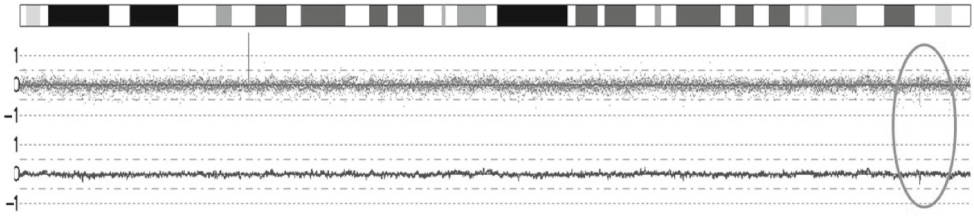
12. Classen,A.K., Bunker,B.D., Harvey,K.F., Vaccari,T., & Bilder,D. A tumor suppressor activity of Drosophila Polycomb genes mediated by JAK-STAT signaling. Nat. Genet. 41, 1150-1155 (2009).

13. Martinez,A.M. et al. Polyhomeotic has a tumor suppressor activity mediated by repression of Notch signaling. Nat. Genet. 41, 1076-1082 (2009).

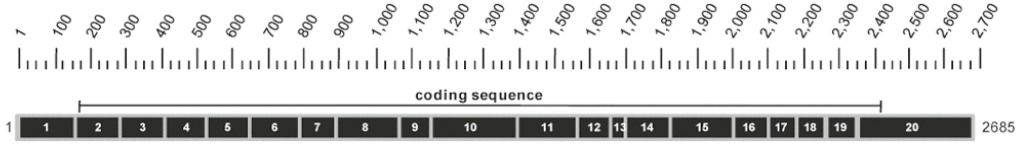
14. Simon,J.A. & Lange,C.A. Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat. Res. 647, 21-29 (2008).

Supplementary information

Supplementary Figure 1: 250k SNP array profile of chromosome 7 showing a microdeletion at 7q36.1.
Genomic profile of chromosome 7 in subject 87 showed a microdeletion located on 7q36.1 (red circle). This microdeletion encompassed the CUL1 and EZH2 genes (see also Figure 1a). Relative intensities of individual (red dots) and 10 consecutive SNPs (blue line) on chromosome 7 are indicated. For this patient, no T cells could be obtained to show that the deletion was acquired. However, this region was never found to be affected in a large control cohort of 1015 healthy controls that were individually hybridized.



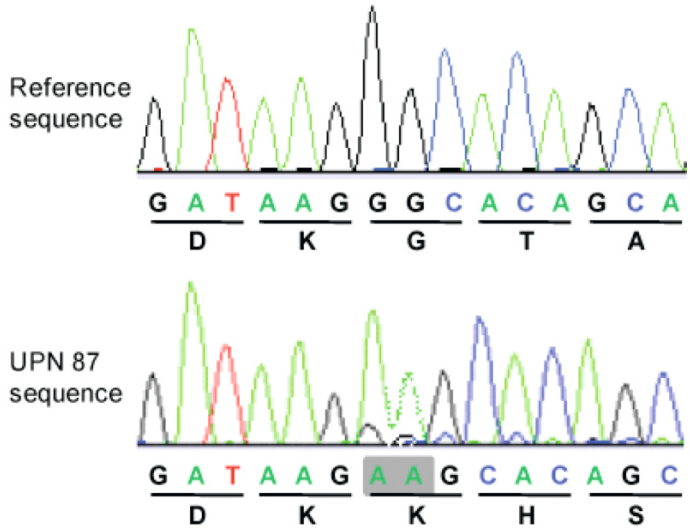
Supplementary Figure 2: EZH2 exon organization and sequence primers.
Exon organization of EZH2 according to GenBank: EZH2 isoform 1, NM_004456.3, representing the longest isoform. Localization of the primers is indicated in the table. M13 sequence primers are indicated in lower case.



Exon	Amplification primer sequence (5' to 3')	Forward / Reverse	Location	Sequence primersequence (5' to 3')
2	tgtaaaacgacggccagtAAGTGTTTTAAGGATTTAACCATGCA	Forward	intron 1	tgtaaaacgacggccagt
	caggaaacagctatgaccCCTTTATATTTAGGGAGGCATTTCTG	Reverse	intron 2	caggaaacagctatgacc
3	tgtaaaacgacggccagtGTCAACAAAGAAAGGATGGCAAT	Forward	intron 2	AAGAGTATGTTTAGTTCCA
	caggaaacagctatgaccAGCTAATAGTGTGATCTACAGCAGTCATT	Reverse	intron 3	caggaaacagctatgacc
4	tgtaaaacgacggccagtGGGTAGGCAGCATCTCTTTACAATAT	Forward	intron 3	tgtaaaacgacggccagt
	caggaaacagctatgaccAATTTATACTGTCTTGATTCACCTTGACA	Reverse	intron 4	caggaaacagctatgacc
5	tgtaaaacgacggccagtTTTTCTCAGATATGCTTATTGGTGAGA	Forward	intron 4	tgtaaaacgacggccagt
	caggaaacagctatgaccGTTCAGTCCCTTATAGTTCAGTGCAA	Reverse	intron 5	caggaaacagctatgacc
6	tgtaaaacgacggccagtTTGCCTAACACCAGTCCTGAAA	Forward	intron 5	tgtaaaacgacggccagt
	caggaaacagctatgaccTCCCAAGTGCTAGGATTACAGAGTTAG	Reverse	intron 6	caggaaacagctatgacc
7	tgtaaaacgacggccagtGTAGCAGAGCTGGGAGTAGAACCTA	Forward	intron 6	tgtaaaacgacggccagt
	caggaaacagctatgaccGTAATGCAGAGTACCACAAGTACACATG	Reverse	intron 7	caggaaacagctatgacc
8	tgtaaaacgacggccagtAATTTGATTCTTGATAACACCATGCA	Forward	intron 7	tgtaaaacgacggccagt
	caggaaacagctatgaccCAGAGCAATCCTCAAGCAACAAA	Reverse	intron 8	caggaaacagctatgacc
9	tgtaaaacgacggccagtGAGGAGGAATGGAGAATACGTTGT	Forward	intron 8	tgtaaaacgacggccagt
	caggaaacagctatgaccGAAACAGCATGGGTGAGAAAGC	Reverse	intron 9	caggaaacagctatgacc
10	tgtaaaacgacggccagtATGAGTTTTAGAACTTTGCCCTGATG	Forward	intron 9	tgtaaaacgacggccagt
	caggaaacagctatgaccCACAACACGAACTTTCACAGAACAG	Reverse	intron 10	caggaaacagctatgacc
11	tgtaaaacgacggccagtTGTGAACTACGATGGGTTAGTGTTTT	Forward	intron 10	tgtaaaacgacggccagt
	caggaaacagctatgaccTTAAAAGTCTACATTGGGGAAATTCTG	Reverse	intron 11	caggaaacagctatgacc
12	tgtaaaacgacggccagtGCATCACAGTTCTTGACCAGAATATAA	Forward	intron 11	tgtaaaacgacggccagt
	caggaaacagctatgaccAGTTCTGTTTTTGATGGCAGTTTAAG	Reverse	intron 12	caggaaacagctatgacc
13	tgtaaaacgacggccagtAGCCTGAGCCATCAAGCTGTT	Forward	intron 12	tgtaaaacgacggccagt
	caggaaacagctatgaccCCAGTCAGCCTCCACTTTACAGA	Reverse	intron 13	caggaaacagctatgacc
14	tgtaaaacgacggccagtCCGTGCTTTAGATGGAACTCATAA	Forward	intron 13	tgtaaaacgacggccagt
	caggaaacagctatgaccGCCTCACACACACAGACACACA	Reverse	intron 14	caggaaacagctatgacc
15	tgtaaaacgacggccagtCGCTGTTAATACTTCCATTTCCTTGT	Forward	intron 14	tgtaaaacgacggccagt
	caggaaacagctatgaccACACTTTCTCATCAGTTGCACCTTT	Reverse	intron 15	caggaaacagctatgacc
16	tgtaaaacgacggccagtACAGGTCTGAGGATTTACAGTGATAGC	Forward	intron 15	tgtaaaacgacggccagt
	caggaaacagctatgaccCAGTTTATGGCAATTCATTTCCAAT	Reverse	intron 16	caggaaacagctatgacc
17	tgtaaaacgacggccagtTGGGAAAGAGAACTTGGCTGTAGT	Forward	intron 16	tgtaaaacgacggccagt
	caggaaacagctatgaccCCAGTTCCTTTCAAGCAAGCA	Reverse	intron 17	caggaaacagctatgacc
18	tgtaaaacgacggccagtAACAATAGTGTGTTCTTCCAAATGTCA	Forward	intron 17	tgtaaaacgacggccagt
	caggaaacagctatgaccCTGGTGTCAGTGAGCATGAAGAC	Reverse	intron 18	caggaaacagctatgacc
19	tgtaaaacgacggccagtCCTTTGACGTGAGAATTGGAACT	Forward	intron 18	tgtaaaacgacggccagt
	caggaaacagctatgaccCTGTCAACAGCAGGGTGAGAAAT	Reverse	intron 19	caggaaacagctatgacc
20	tgtaaaacgacggccagtACCCACTATCTTCAGCAGGCTTT	Forward	intron 19	TCAGCACATGTTGGATGG
	caggaaacagctatgaccCTTCCACATATTCACAGGCAGTATTAGT	Reverse	downsteam of exon 20	caggaaacagctatgacc
				CAGCTGTTTCAGAGGAGGGGG

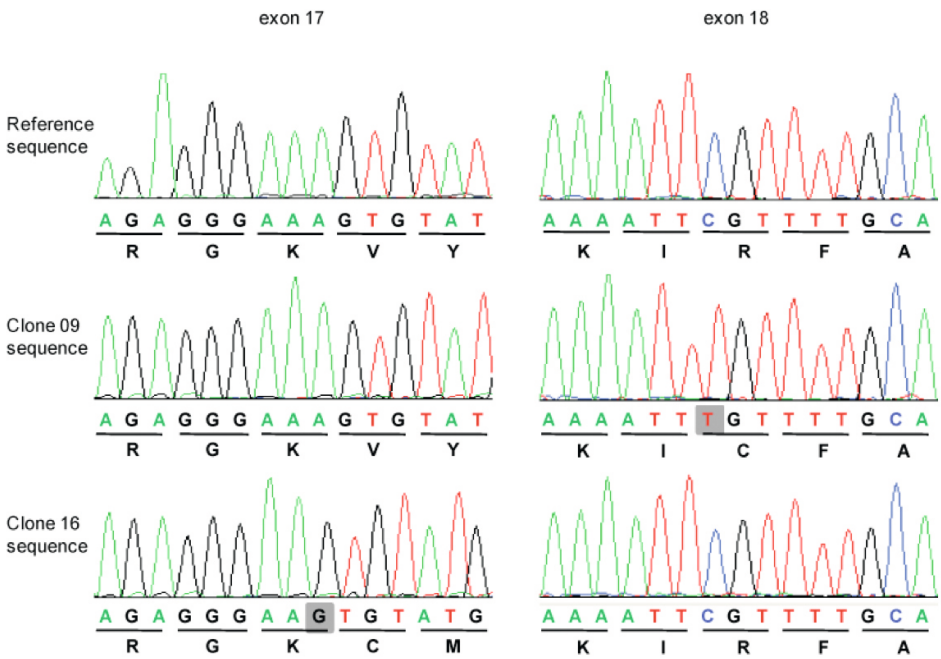
Supplementary Figure 3: Sequence analysis of genomic DNA from subject 87.

*SNP array analysis revealed a 130-kb microdeletion at 7q36.1, containing the CUL1 and EZH2 genes, in subject 87 (**Fig. 1a**). Subsequent sequence analysis of genomic DNA from this subject showed a c.703delGinsAA mutation in the remaining copy of EZH2, predicting the formation of a truncated protein.*



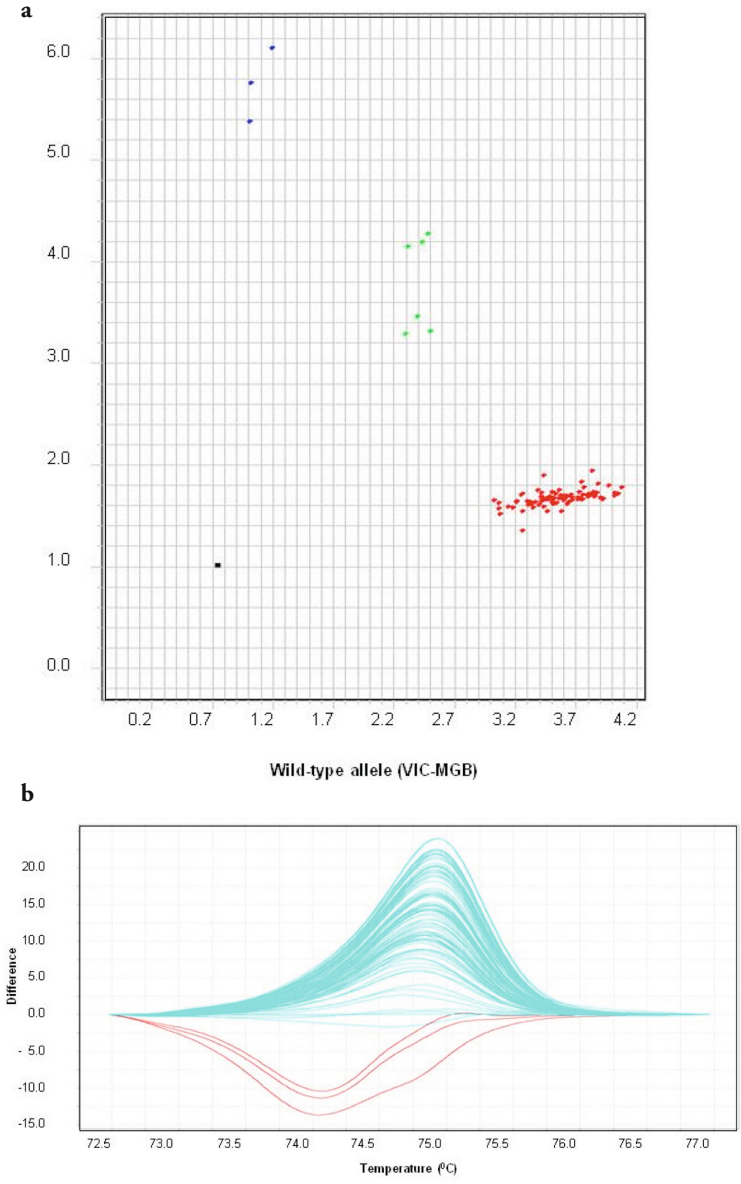
Supplementary Figure 4: Bi-allelic point mutations in subject 96.

A PCR product covering EZH2 exons 17 and 18 from subject 96 was cloned and individual clones were sequenced (Supplementary Methods online). Clones contained either the c.1983delA mutation in exon 17 (e.g. clone 16) or the c.2068C>T mutation in exon 18 (e.g. clone 9), indicating the bi-allelic nature of these mutations. Mutant nucleotides are marked.



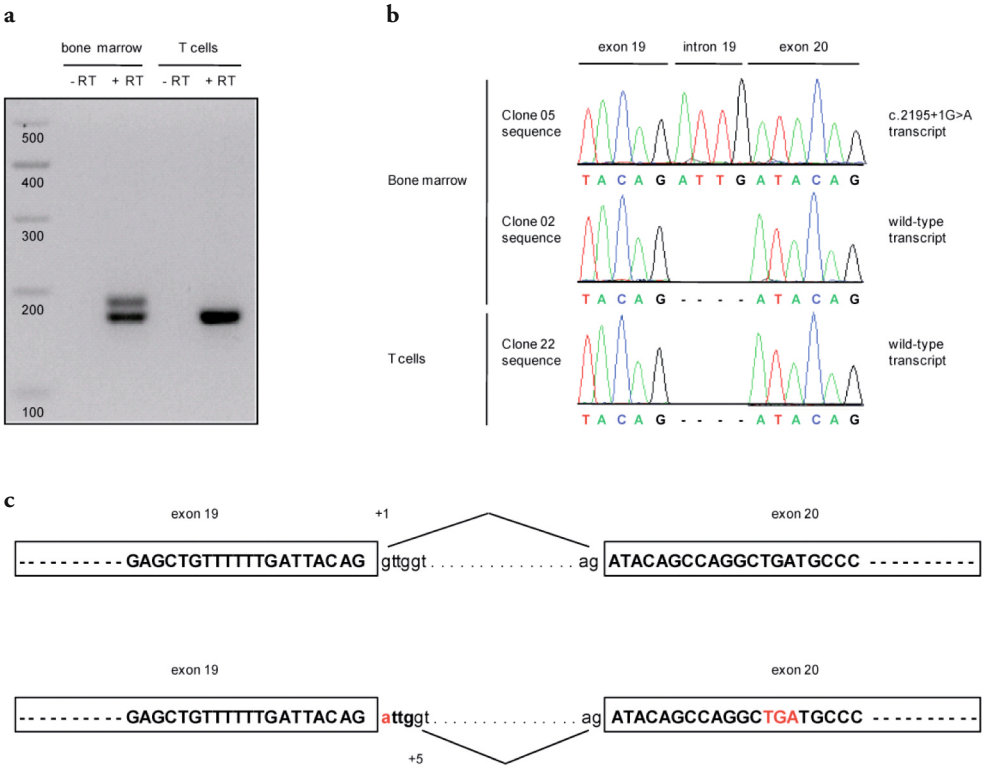
Supplementary Figure 5: Missense mutations are absent in healthy donors.

*Allelic discrimination assays (***Supplementary Table 2** *and* **Supplementary Methods** *online) were performed to screen genomic DNA from white blood cells of healthy donors (n = 250).* **(a)** *Representative result of allelic discrimination assays (PCR with allele-specific probes). The missense variant c.2025C>A (exon 17, subject 97) is absent in 250 healthy donors as determined by this assay. Blue dots represent subject 97 carrying this mutation (triplicate). Red dots represent 80 healthy donor samples and two MDS patients without this mutation (subjects 63 and 73, both in triplicate). Green dots represent 1:1 DNA mixtures of subject 97 and subject 63 (triplicate) and of subject 97 and subject 73 (triplicate) creating artificial heterozygotes. The black dot represents the No Template Control. Variants were auto-called by the SDS 2.3.2. allelic discrimination program (Applied Biosystems).* **(b)** *Representative result of allelic discrimination assays (PCR followed by HRM analysis). The missense variant c.2068C>T (exon 18, subject 96) is absent in 250 healthy donors. Blue lines represent 83 healthy donor samples and two MDS patients without this mutation (subject 63 and subject 73, both in triplicate). Red lines represent subject 96 carrying this mutation. Variants were auto-called by the HRMv1.0 software (Applied Biosystems).*



Supplementary Figure 6: Expression of wild-type and mutant alleles in subjects 10 and 127.

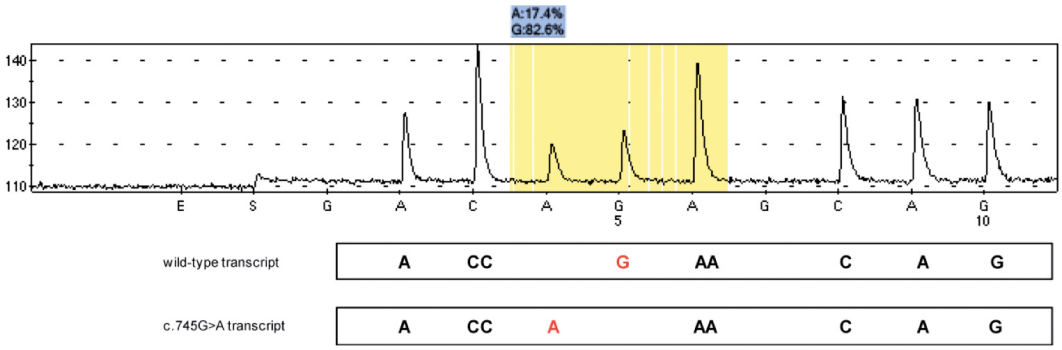
(a-h) *Expression of wild-type and mutant alleles in subjects 10 and 127.* **(a-c)** *Subject 10 harbored a heterozygous mutation c.2195+1G>A in the donor splice site of intron 19. This resulted in aberrant mRNA splicing as shown by agarose gel electrophoresis and sequence analysis of RT-PCR products (***Supplementary Methods** *online).* **(a)** *Bone marrow cDNA from subject 10 was amplified using primers directed against the exon 18/19 boundary and exon 20. The PCR product demonstrated the presence of two different transcripts. Amplification of cDNA from the non-neoplastic T cells showed the presence of only one transcript.* **(b)** *Sequence analysis of the cloned PCR products revealed an aberrant transcript in bone marrow cells, containing an insertion of four intronic base pairs G>A, T, T and G between exon 19 and 20. Wild-type transcripts were present in both bone marrow cells and non-neoplastic T cells.* **(c)** *Wild-type RNA was spliced at the r.2195+1 donor splice site. Mutant RNA was aberrantly spliced at r.2195+5.*



(d-e) *Subject 10 harbored the heterozygous missense mutation c.745G>A in exon 8. Transcripts carrying this mutant were expressed as determined by pyrosequencing (***Supplementary Methods** *online).* **(d)** *Bone marrow cDNA from subject 10 was amplified using primers directed against exon 7 and exon 8 (green arrows), spanning the c.745G>A mutation (indicated in red). Pyrosequencing of the PCR products was performed using a nested primer directed against the exon 7/8 boundary (yellow arrow).* **(e)** *Pyrosequence analysis revealed the presence of both wild-type transcripts (83 %) and mutant transcripts (17 %), as determined by the Genescan software.*

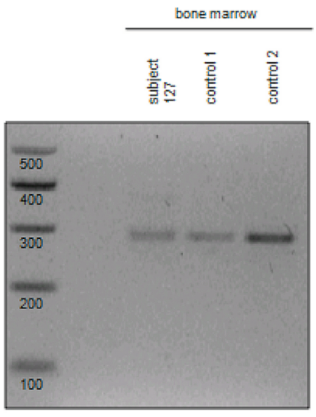
d

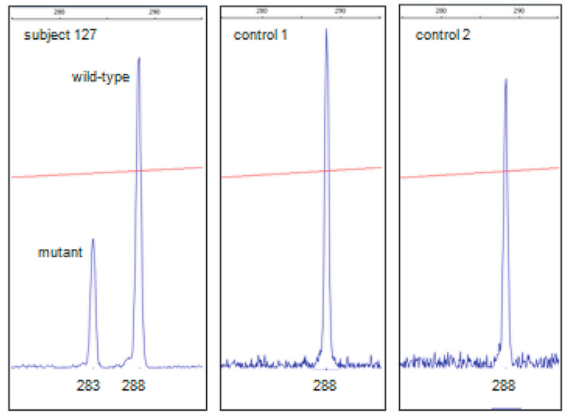
e



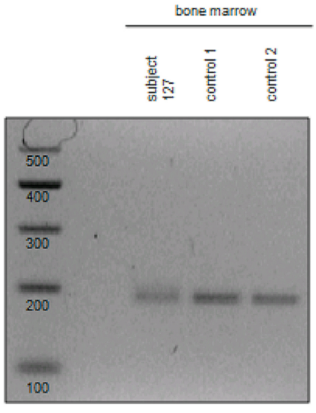
(f-h) *Subject 127 carried the heterozygous deletion c.1212-1216delGAAGA in exon 10 resulting in a frameshift. Transcripts carrying this mutant were expressed as determined by Genescan and sequence analysis of RT-PCR products (***Supplementary Methods** *online).* **(f)** *Bone marrow cDNA from subject 127 was amplified using primers directed against exon 9 and exon 11. Genescan analysis unveiled the presence of two different transcripts: a wild-type transcript of the expected 288 bp and a mutant transcript of 283 bps (ratio = 2.5 : 1). Genescan analysis of amplified cDNA from bone marrow cells of two healthy donors (controls 1 and 2) showed the presence of the wild-type transcript only.* **(g)** *Bone marrow cDNA from subject 127 was amplified using primers directed against exon 10 and exon 11. Genescan analysis detected two different transcripts: a wild-type transcript of the expected 188 bp and a mutant transcript of 183 bp (ratio = 1.8 : 1). Genescan analysis of cDNA from bone marrow cells of two healthy controls showed the presence of the wild-type transcript only.*

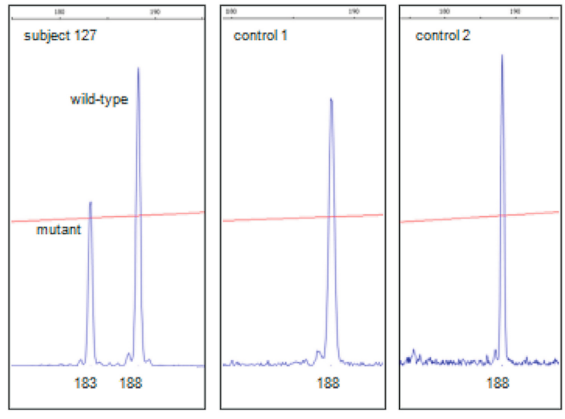
f



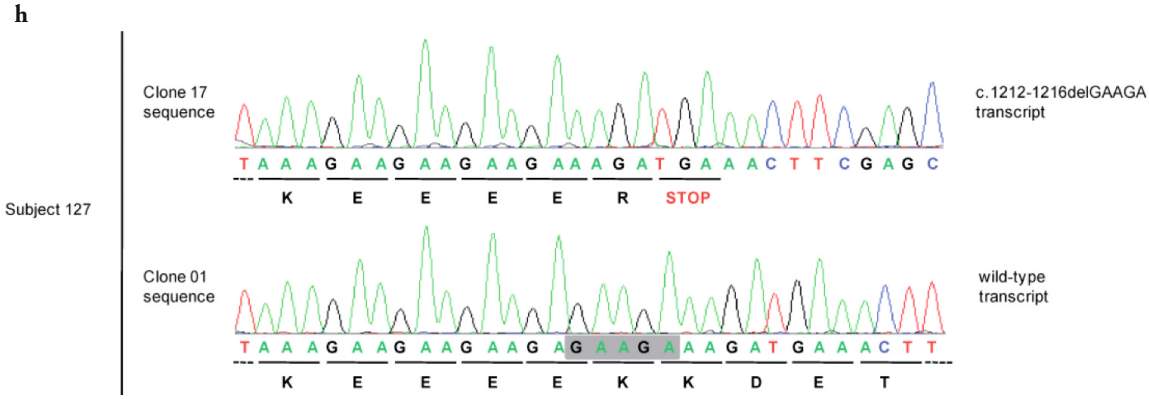


g

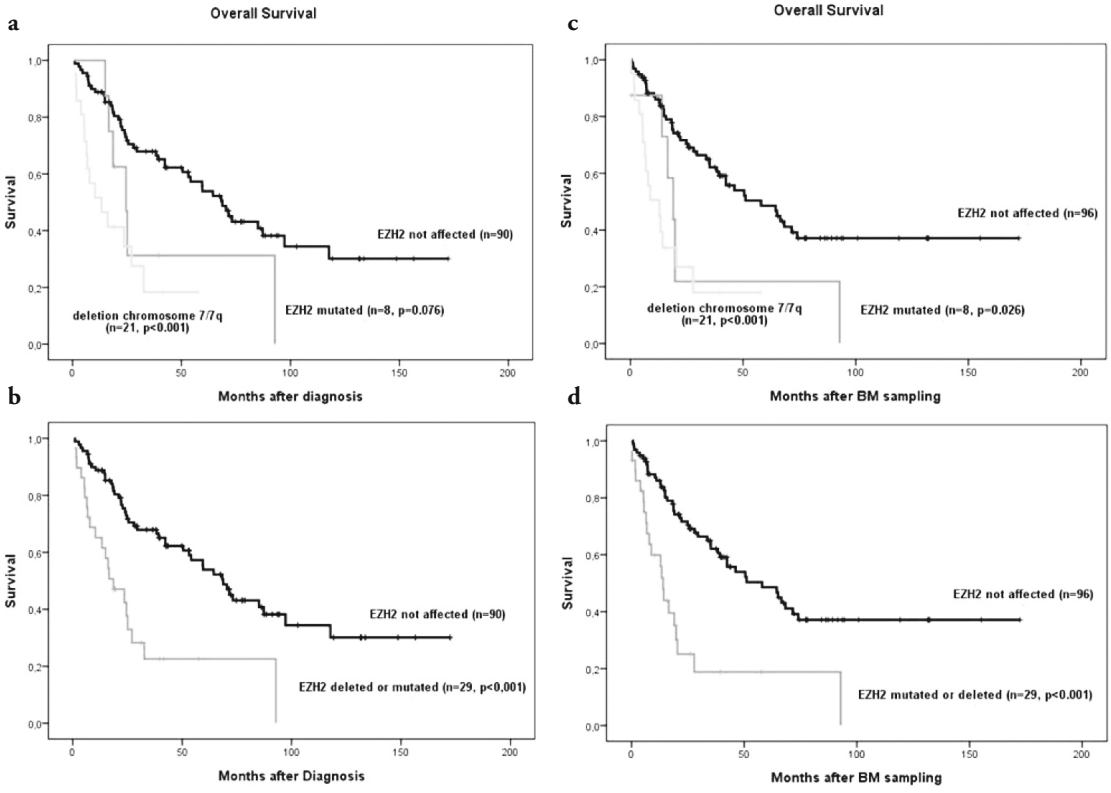




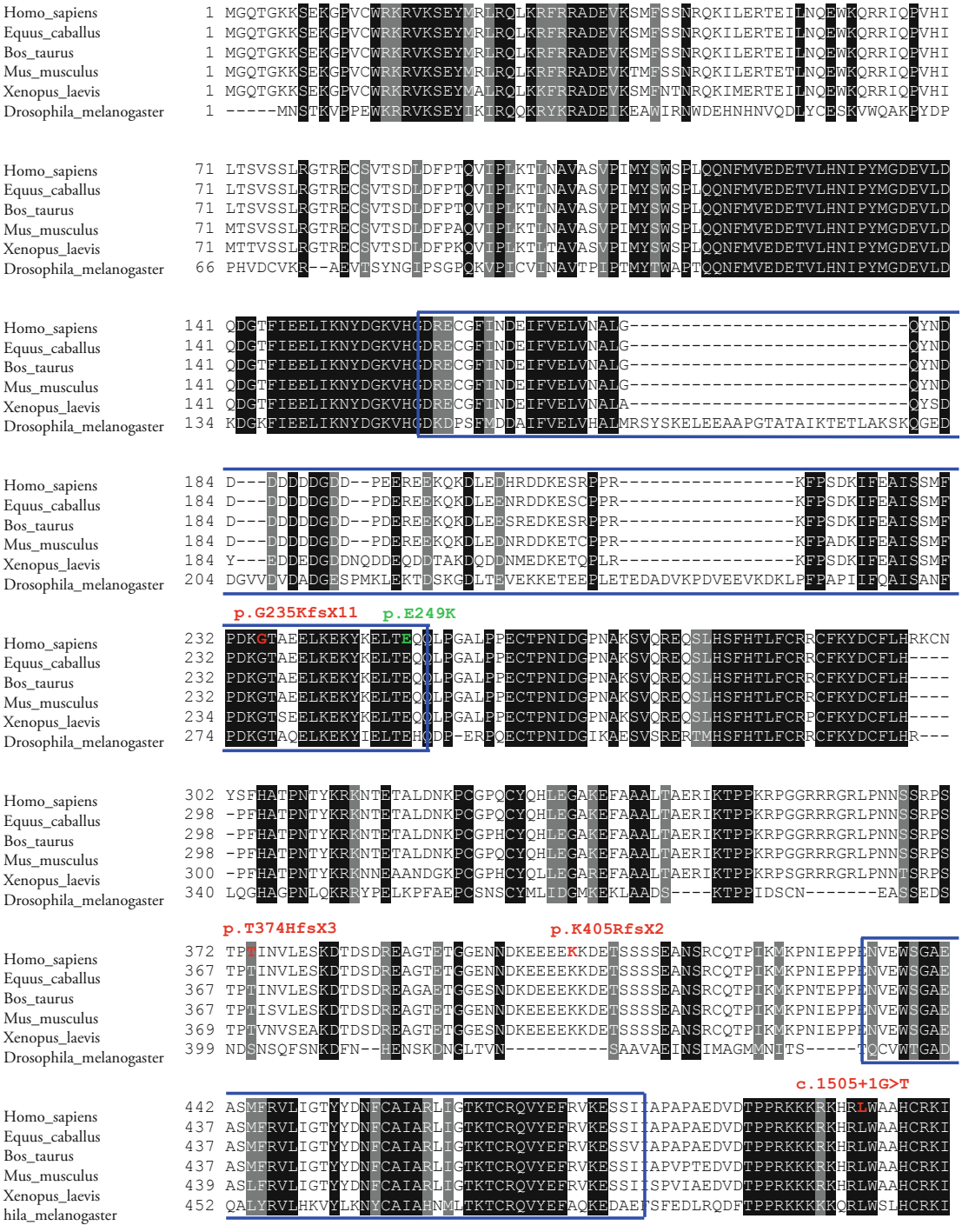
(h) *Sequence analysis of the cloned PCR products confirmed the presence of two different transcripts in the bone marrow cells of subject 127. The deleted nucleotides are marked.*

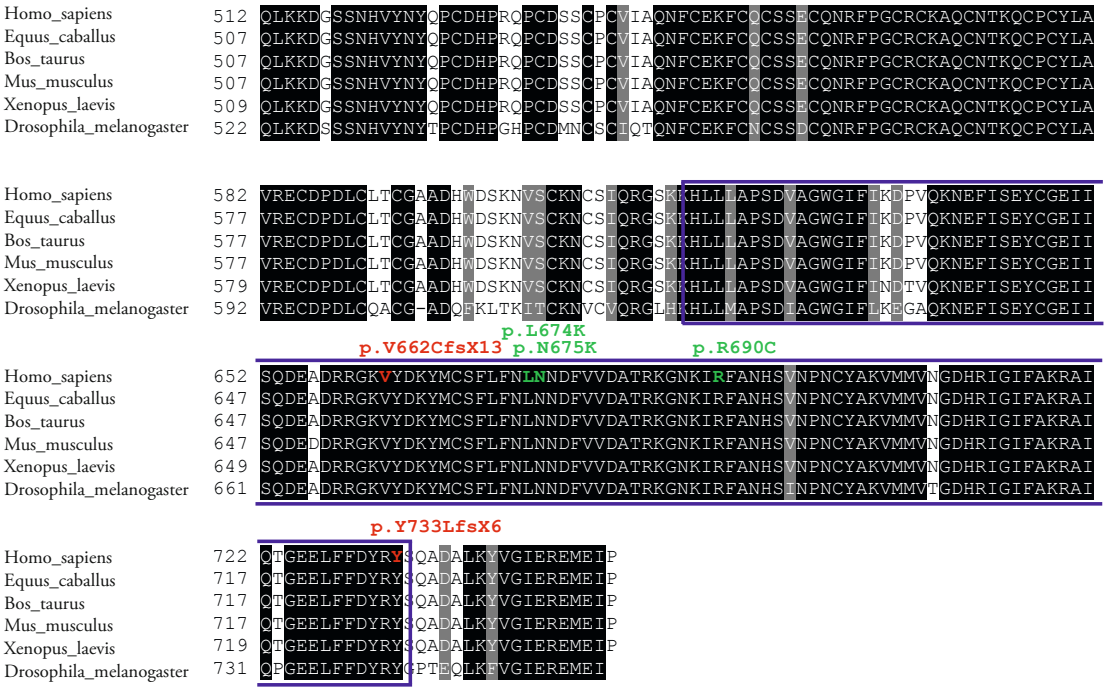


Supplementary Figure 7: Aberrations of 7q36.1 correlate with poor survival in MDS.
Kaplan-Meier analysis showing overall survival of MDS patients with and without EZH2/7q36.1 aberrations. Since diagnosis material was not available in a subset of patients, overall survival was calculated from the date of diagnosis **(a** *and* **b)** *as well as from the date of bone marrow (BM) sampling* **(c** *and* **d).** *p-values were calculated comparing patients with a deletion or mutation of EZH2 to patients in whom EZH2 was not affected.*



Supplementary Figure 8: Alignment of orthologous EZH2 proteins and summary of point mutations.
Alignment of EZH2 orthologs in various species. Blue boxes represent two the SANT domains and the SET domain respectively. Positions of donor splice site and frameshift mutations are indicated in red. In case of donor splice site mutation c.1505+1G>T, the first amino acid of the next exon is highlighted in red, since the deduced protein sequence is unknown. Missense mutations are indicated in green.





Supplementary Methods

Study participants

The characteristics of MDS patients (n = 102), the collection of blood and/or bone marrow after obtaining informed consent and the isolation of cells have been described previously[3]. The characteristics of 24 additional MDS patients are described in this paper (**Supplementary Table 1** online). Consecutive subjects from the Radboud University Nijmegen Medical Center (n = 107) belonging to all different WHO and IPSS categories and cytogenetic subgroups were included, if bone marrow was available, as well as a second cohort of karyotypically normal cases from the University Hospital Leuven (n = 19). For control experiments, blood from 250 healthy individuals and bone marrow from two healthy donors was obtained after informed consent. Cells were frozen in liquid nitrogen until further use. For SNP array analysis and genomic sequencing, DNA was extracted from FACS-sorted lymphocyte-depleted bone marrow (n = 41) and Ficoll density gradient–isolated mononuclear cells from bone marrow (n = 85). DNA from healthy donors (controls, n = 250) was isolated from white blood cells after NH_4Cl lysis. RNA from healthy donors (controls, n = 2) was isolated from total bone marrow as described.

Supplementary Table 1: Characteristics of MDS patients UPN106 - UPN129.

UPN	FAB	WHO	IPSS	Karyotype	TET2 mutation
106	RA	RA	int-2	45,XY,-7[6]/46,XY[4]	no
107	RARS	RCMD-RS	int-1	46,XX,t(3;3)(q21;q26)[10]	no
108	RAEB	RAEB-1	int-1	46,XX,del(5)(q12q33), add(7)(q11), del(8)(q22), add(9)(q34), ?10 [6]/ 46,sl,add(14)(q32), add(19)(p13) [3]/46,XX[1]	no
109	RA	RCMD	int-1	45,X,-Y [4]/46,XY[16]	no
110	RARS	RCMD-RS	int-1	normal	yes
111	RA	5q-	unknown	46,XY, 5q-	no
112	RARS	RCMD-RS	int-1	45,XX,del(5)(q13q33),-7 [5]/ 45,XX,idem,del(4)(q25q31),-17,der(20)t(17;20) (q12;q13.1),+mar[5]/46,XX[11]	no
113	RAEB	RAEB-1	int-2	45,XX, -7 [8]/46,XX[2]	no
114	RA	RCMD	int-2	46,XY,der(7)t(1;7)(p10;q10)[9]/46,XY[1]	yes
115	RAEB	RAEB-2	int-2	normal	no
116	RA	RA	int-1	normal	no
117	RAEB-t	RAEB-2	int-2/high	no metaphases, FISH: no deletion 5/7 or trisomy 8/21	no
118	RA	RCMD	low	normal	no
119	RAEB	RAEB-1	int-2	46,XX,del(5)(q15q33),der(7)del(7)(p11)add(7) (q3?3) [8]/46,XX [2]	no
120	RARS	RARS	low	normal	no
121	RAEB	RAEB-1	int-2	44~47,XY,-2,-2,-4, del(4)(q31),-5,-7,+15,-17,del(20)(q11), +mar1, +mar2,+mar3 [cp2]/ 44,sl.add(9)(q34),add(12)(p13),-13,-15,-15,-16,add(17)(p13), +mar4,+mar5,+mar6,+mar7 [cp6]/46XY [2]	yes
122	RARS	RARS	low	normal	no
123	RA	RCMD	int-1	46,X,t(Y;11;20)(q11;p15;p11)[10]	no
124	RA	5q-	int-1	46,XX,del(5)(q12q33) [7]/46,XX [3]	yes
125	RAEB	RAEB-1	int-1	normal	yes
126	RA	RCMD	int-2	47,XX,-7,+21,+22[5]	no
127	RA	RA	low	normal	no
128	RAEB	RAEB-1	int-2	46,XY,-3,add(5)(q?31),der(9)t(3;9)(q13;q22), add(21)(p11), add(22)(q13),+mar1 [16]/ 47,sl,+mar2 [4]	no
129	RAEB-t	RAEB-2/ AML	int-2/high	46,XX,del(5)(q22q33)[9]/46,XX[1]	no

SNP array analysis

The SNP array analysis of genomic DNA from 102 MDS patients was performed and described previously[3].

Sequence analysis

DNA was extracted using Qiagen spin-columns and DNA quality was checked by gel analysis and nanodrop ND-1000 spectrophotometer analysis. We conducted bidirectional sequence analysis on PCR-amplified genomic DNA fragments spanning the entire coding region and splice sites (**Supplementary Fig. 3** online). Sequence variations were confirmed by independent PCR-amplification and sequencing of the original DNA. To analyze whether the observed variants were acquired, DNA from cultured non-neoplastic T cells was amplified and sequenced, whenever available (n = 3). To minimize the chance that the remaining putative variants represent SNPs, we compared the observed variants with public databases (NCBI SNP and EST database) and investigated the presence of missense variants in healthy donors (n = 250) by allelic discrimination assays. Detected mutations were described according to the nomenclature explained at http://www.hgvs.org/mutnomen[5].

Cloning of variant sequences

Subject 96 harbored two heterozygous variants (c.1983delA in exon 17 and c.2068C>T in exon 18) in close proximity, enabling DNA amplification using primers spanning both variants: forward primer 5' - tgtaaaacgacggccagtTGGGAAAGAGAACTTGGCTGTAGT - 3' (intron 16) and reverse primer 5'- caggaaacagctatgaccCTGGTGTCAGTGAGCATGAAGAC - 3' (intron 18). The generated amplicon was cloned into the pDrive cloning vector (Qiagen). To determine whether the variants were mono- or bi-allelic, individual clones were sequenced using primers
5' -TGGGAAAGAGAACTTGGCTGTAGT - 3' (forward primer, intron 16),
5' -CCAGTTCCTTTCAAGCAAGCA- 3' (reverse primer, intron 17),
5' -AACAATAGTGTGTTCTTCCAAATGTCA- 3' (forward primer, intron 17) and
5'- CTGGTGTCAGTGAGCATGAAGAC - 3' (reverse primer, intron 18).

T cell cultures

To obtain DNA from non-malignant cells, we conducted in vitro expansion of polyclonal T cells from frozen blood or bone marrow samples. These samples were depleted of myeloid cells by adherence to tissue culture flasks. The remaining cells were cultured for 7-14 days in IMDM (Gibco Invitrogen Corporation) supplemented with human serum (10%, PAA Laboratories GMBH), IL-2 (100 IU/ml) and CD3/CD28 coated beads (Dynabeads, Invitrogen). The purity of T-cells was determined by FACS analysis of CD3+ cells. DNA was extracted from T cells when the purity was > 90%.

Allelic discrimination assay: PCR with allele-specific probes

We examined DNA obtained from 250 healthy donors for the presence of the identified homozygous missense variant c.2025C>A (subject 97). Probes (**Supplementary Table 2** online) were designed for recognition of the variant (FAM-MGB probes, Applied Biosystems) and wild-type (VIC-MGB probes, Applied Biosystems) sequences. Primers (**Supplementary Table 2** online) spanning the site of variation were used in combination with these probes to generate a PCR product of 115 bp using the 7900HT Fast Real-Time PCR System (Applied Biosystems). MDS samples without this mutation (subjects 63 and 73) and the MDS sample with this homozygous missense mutation (subject 97) were included in each run in triplicate. Artificial heterozygotes were created by 1:1 mixing of DNA from subject 97 and subject 63 (triplicate) and subject 97 and subject 73 (triplicate). End point VIC/FAM measurements were performed and values were corrected for passive reference ROX values. Corrected values were plotted automatically by the SDS 2.3.2. allelic discrimination program (Applied Biosystems).

Supplementary Table 2: Primers and probes for allelic discrimination.

UPN	Variant	Assay	Amplification primer and probe sequence (5' to 3')	Forward / Reverse	Reporter/ Quencher	Wild-type/ Variant	Location
97	c.2025C>A exon 17	Allele-specific probes	GAAGCTGACAGAAGAGGGAAAGTG	Forward			exon 17
			TGCCACATGCAACTCAGGAA	Reverse			intron 17
			TCAACTTGAACAATGGT	Probe	VIC / MGB	Wild-type	exon 17 - intron 17
			TCAACTTGAAAAATGGT	Probe	FAM / MGB	Variant	exon 17 - intron 17
96	c.2068C>T exon 18	HRM	AAATTATTCACTGGGCTGTGCTTACT	Forward			intron 17
			TACCTTTTGCATAGCAGTTTGGATT	Reverse			exon 18 - intron 18

Allelic discrimination assay: PCR followed by high-resolution melting (HRM) analysis.

We examined the DNA of 250 healthy donors for the presence of the newly identified missense variant c.2068C>T (subject 96). Primers (**Supplementary Table 2** online) spanning the site of variation and the MeltDoctor™ HRM Master Mix (Applied Biosystems) were used to generate a PCR product of 123 bp using the 7500 Fast Real-Time PCR System (Applied Biosystems). MDS samples without this mutation (subjects 63 and 73) and the MDS sample containing this heterozygous missense mutation (subject 96) were included in each run in triplicate. Melting temperatures of the generated amplicons were recorded during the subsequent dissociation stage by the 7500 System SDS Software. Variants were auto-called and grouped by the HRMv1.0 software (Applied Biosystems).

Supplementary Table 3: Identified SNPs in the coding sequence and intron-exon boundaries of EZH2 in MDS patients.

Substitution	SNP ID	Type of substitution	Amino acid change	Frequency in MDS cohort (%)	Present in T-cell fraction
c.87C>T		synonymous	p.L29L	1/126 (0,8)	N/A
c.396T>C	rs61732845	synonymous	p.P132P	3/126 (2,4)	N/A
c.553G>C	rs2302427	non synonymous	p.D185H	20/126 (16,0)	N/A
c.623A>G	rs61753264	non synonymous	p.D208G	1/126 (0,8)	N/A
c.933T>C		synonymous	p.Y311Y	1/126 (0,8)	N/A
c.1457C>T		synonymous	p.P486P	2/126 (1,6)	N/A
c.1731G>A	rs41277437	synonymous	p.P577P	7/126 (5,6)	N/A
c.2113-6C >T		non-coding region	non-coding region	2/126 (1,6)	yes
c.2113-9A>G		non-coding region	non-coding region	1/126 (0,8)	yes
c.2276+6T >G	rs41277434	non-coding region	non-coding region	12/126 (9,5)	N/A

RNA isolation and reverse transcriptase reactions.

RNA was extracted from total bone marrow or expanded T cells using TRIzol Reagent (Invitrogen) or RNA-Bee (Bio-Connect). Traces of genomic DNA were depleted from the isolated RNA by DNAse I treatment (Invitrogen) according to the manufacturer's instructions. Reverse transcriptase (+RT) reactions on 1 μg of RNA were performed using M-MLV reverse transcriptase (Invitrogen). Negative controls containing no reverse transcriptase (-RT) were included to detect remaining traces of genomic DNA.

Confirmation of the c.2195+1G>A mutation (subject 10) at the transcript level

Subject 10 carried a heterozygous donor splice site mutation in intron 19 (c.2195+1G>A). To determine the effect on splicing, we designed PCR primers spanning the affected intron: forward primer 5' - CCAAACTGCTATGCAAAAGTTATGAT - 3' (exon 18/19 boundary) and reverse primer 5' - CAGATGTCAAGGGATTTCCATTTC - 3' (exon 20). PCR reactions were performed on cDNA (-RT and +RT). PCR products were analyzed by agarose gel (2%) electrophoresis and by sequence analysis of products that were cloned into the pDrive cloning vector (Qiagen).

Confirmation of the c.745G>A mutation (subject 10) at the transcript level

Subject 10 carried a heterozygous missense mutation in exon 8 (c.745G>A). To determine the presence of this mutation in RNA transcripts, we designed primers for amplification and pyrosequencing of cDNA, spanning the c.745G>A mutation:
forward amplification primer (exon 7)
5' - AATGTTTCCAGATAAGGGCACAG - 3'
reverse amplification primer (exon 8)
5' - TTAGCATTTGGTCCATCTATGTTG - 3'
forward pyrosequencing primer (exon 7/8 boundary)
5' - AGGAAAAATATAAAGAACTC - 3'

Confirmation of the c.1505+1G>T mutation (subject 73) at the transcript level

Subject 73 carried a donor splice site mutation in intron 12 (c.1505+1G>T). To determine the effect on splicing, we designed two PCRs. For the 1st PCR, we used primers spanning the affected intron: forward primer 5' - GGACCAAAACATGTAGACAGGTGTA - 3' (exon 11/12 boundary) and reverse primer 5' - GCAGTGTGCAGCCCACAA - 3' (exon 13). For the 2nd PCR, we used primers spanning the entire 3' coding region (exon 11/12 to exon 20): forward primer 5' - GGACCAAAACATGTAGACAGGTGTA - 3' (exon 11/12 boundary) and reverse primer 5' - CAGATGTCAAGGGATTTCCATTTC - 3' (exon 20). PCR reactions were performed on cDNA (-RT and +RT). PCR products were analyzed by agarose gel (2%) electrophoresis and by sequence analysis of PCR products.

Confirmation of the c.1212-1216delGAAGA mutation (subject 127) at the transcript level

Subject 127 harbored a heterozygous c.1212-1216delGAAGA mutation in exon 10. To assess the presence of this mutation, we designed two PCR assays spanning the affected exon:
forward primer A 5' - GACCACAGTGTTACCAGCATTTG - 3' (exon 9), forward primer B 5'-AAGAGGACGGCTTCCCAATAA-3' (exon 10) and reverse primer 5'-TGTTTGACACCGAGAATTTGCT - 3' (exon 11, coupled to a 5' 6FAM label). PCR reactions were performed on cDNA (-RT and +RT). PCR products were analyzed by agarose gel (2%) electrophoresis and by sequence analysis of products that were cloned into the pDrive cloning vector (Qiagen). GeneScan analysis was performed using the 6FAM-coupled PCR products in combination with the GeneScan™ 500 LIZ® Size Standard (Applied Biosystems) on a 3730 DNA Analyzer (Applied Biosystems).

Chapter 6

Overview and Discussion

Myelodysplastic syndromes are known as a group of heterogeneous hematopoietic stem cell disorders, sharing a dysplastic morphology of erythroid, myeloid or megakaryocytic cells. Multiple classification systems are currently in use, or are being developed, to define distinctive subgroups sharing similar characteristics. These classification systems are primarily based on morphological features, not on differences and similarities in the pathogenesis of the various disease subtypes. Finding specific genes or groups of genes that are commonly affected in patients with MDS provides a way to gain more insight into the origin of these diseases and eventually to develop novel, targeted therapies.

Gene expression analysis

In trying to define genes involved in MDS pathogenesis, analysis of mRNA expression patterns has drawn a lot of attention. These studies took a flight after the development of micro-array technologies, enabling researchers to study the expression of thousands of genes at the same time. It has led to the identification of commonly deregulated pathways in MDS, but it has proven difficult to pinpoint how these pathways exactly contribute to the development of this disease. In contrast to examining global gene expression, the research described in **chapter 2** of this thesis focuses on genes involved in apoptosis, a process which has often been implicated in the pathogenesis of MDS. We aimed at finding similarities between different groups of MDS patients. We restricted the analysis to apoptosis-related genes, used a microfluidic card platform, which is more sensitive than micro-array analysis, and studied three hematopoietic cell populations. In contrast to our expectations, distinct expression patterns distinguishing patients from healthy controls or patients with indolent and aggressive forms of MDS were not seen. The activity of single genes, such as *BIK*, could play a role in defining different MDS subgroups, but this would require further research. Moreover, studying expression levels of genes may increase insight into pathways that are important in MDS pathogenesis, but it does not answer the question what mechanisms lie beneath these differences in gene expression.

Using SNP-arrays to detect genomic aberrations

In the second part of this thesis, a different strategy has been used to define common aberrations in MDS patients. The study described in **Chapter 3** focuses on finding commonly occurring DNA aberrations in MDS. Single Nucleotide Polymorphism (SNP) arrays served as a novel tool to study the MDS genome with a higher resolution than had ever been possible before. The aim of performing SNP-array analysis in MDS patients was to find small, commonly affected genomic regions, which would subsequently lead to the identification of individual genes involved in the pathogenesis of MDS. Before focusing on novel chromosomal lesions, we first studied the ability of the array technology to detect the known ones. SNP-arrays do not allow the detection of balanced chromosomal abnormalities, except copy number (CN) neutral loss of heterozygosity (LOH). Thus, translocations or inversions found by karyotyping in our cohort could not be confirmed by SNP-

array analysis. In some cases, however, one of the genes involved in the translocation could be identified because of a microdeletion at the breakpoint (i.e. *ETV6* on chromosome 12 in UPN2 and, likely, *MDS1* on chromosome 3 in UPN40). In about 25% of the patients one or more of the monosomies or trisomies detected by karyotyping could not be confirmed by SNP-array analysis. This applied in particular to trisomies and other aberrations that were only present in a subclone of the metaphases analyzed. Taking these observations into consideration, SNP-array based genomic profiling cannot be regarded as a complete replacement of karyotype analysis with regard to facilitating diagnosis of MDS. However, the capacity of SNP-arrays to detect small deletions and amplifications and CN neutral LOH complements the information obtained by karyotype analysis. Thus, SNP-arrays may facilitate the diagnosis and prognostic evaluation of MDS patients, but the impact on clinical decision making (e.g. should patients with a CN neutral LOH be treated similar to patients with a deletion of 7q) remains, as yet, to be determined. In some cases it proved to be difficult to compare SNP-arrays and karyotyping because of the presence of marker chromosomes in the karyotype. In addition, chromosomal aberrations may have been missed due to 'contamination' of the samples with non-malignant cells. The SNP-array analysis revealed a large number of novel, potentially interesting affected chromosomal regions. Frequently recurring novel micro-deletions or amplifications, as have been described in SNP-array analysis of other hematopoietic malignancies, were not found. But, by combining the results of patients with large and small genomic aberrations, we were able to identify the 4q24 and 7q36 regions, leading to the identification of mutations in the *TET2* and *EZH2* genes (**chapters 3 and 5**). Of note, the smallest-affected-area that led to these genes, was in both cases determined by a single patient, whilst the others carried large chromosomal abnormalities. The possible consequences of aberrations in the *TET2* and *EZH2* genes are discussed in the next section. It is not possible without further research to conclude which of the other novel regions may be implicated in the pathogenesis of MDS. We attempted to exclude non relevant regions by comparing the data with a large panel of controls and the database of normal variants to exclude neutral copy number variations (CNVs). We did not include non-malignant cells (such as fibroblasts) in the analysis of the bone marrow sample of each individual patient. This may have facilitated the discovery of relevant defects. Lymphocytes are in general not regarded as part of the malignant clone in MDS and we used these as a control in a subset of patients. However, as discussed in the next section, it has been published that (a subclone of) lymphocytes may carry the same genetic abnormality as the myeloid cells, thus complicating the interpretation of the relevance of possible aberrations found in both cell populations. Perhaps even more difficult than determining the disease-specificity of novel aberrations is to determine the extent to which they contribute to disease pathogenesis. A substantial part of MDS patients showed complex chromosomal abnormalities. Some of these may have been early events in the pathogenesis of the disease, whereas others may have arisen due to genetic instability of the malignant MDS clone. To distinguish these so called driver and passenger defects requires extensive functional studies. Thus, the interpretation of the importance of novel genomic aberrations requires careful evaluation.

Copy number (CN) neutral loss of heterozygosity (LOH): which regions count?

CN neutral LOH of partial or entire chromosomes may contribute to the development of MDS, since this may lead to homozygosity of mutated genes or transcriptionally repressed or activated (e.g. hyper- or hypomethylated) genes. The frequency of partial CN neutral LOH reported in MDS differs. When first performing SNP-array analysis we included areas consisting of ≥ 70 consecutively homozygous SNPs. In this way, many areas of CN neutral LOH were found, however, in those patients of whom T-cells were available, these areas appeared to be identical. Also, small areas of CN neutral LOH were frequently found in our healthy controls. We also assessed whether certain chromosomal regions are more prone to homozygosity in MDS patients compared to controls by comparing the frequency with which (areas of 10 consecutive) SNPs showed homozygosity. This did not lead to the identification of single affected genes. In other studies, different criteria have been used to define potentially relevant areas of CN neutral LOH. These vary from only the telomeric regions to areas based on their size, e.g. >2Mb. In our final analysis, described in **chapter 3**, we included only large telomeric regions of CN neutral LOH. Concerning the areas of CN neutral LOH on chromosomes 4 and 17, we have shown that they contain the homozygously mutated *TET2* and *TP53* genes. The CN neutral LOH detected on chromosome 7 led to the identification of homozygous mutations in *EZH2*, though not in all patients. We did not focus on the affected regions on chromosomes 1 and 6, although aberrations in these regions have been found by others as well. To answer the question what the role of, in particular, the segmental non-telomeric regions of CN neutral LOH is in the pathogenesis of MDS, analysis of matched malignant and non-malignant cells of the patients is again essential.

TET2 mutations in malignant hematopoiesis

Using SNP-array technology we identified a commonly affected area in MDS patients on chromosome 4q24 encompassing the *TET2* and *PPA2* genes (described in **chapter 3**). *TET2* was shown to be mutated on the remaining allele of patients carrying a 4q24 deletion and also in a substantial number of patients without 4q24 copy number abnormalities. Overall, *TET2* aberrations were detected in 26% of patients with MDS. Studies performed by other groups have reported an 11-23% incidence of *TET2* mutations in MDS (**Table 1**)[1-5]. Notably, it has recently been reported that *TET2* mutations can be detected in T-cells of MDS patients as well, though at a lower level than in CD34+ cells or whole bone marrow[5]. Thus, the incidence of *TET2* mutations in our and other patient cohorts may be an underestimation. We found that *TET2* mutations occurred most frequently in patients with MDS categorized as IPPS category low or intermediate 1, although the difference was not statistically significant. Other groups have shown different patterns of distribution, although all report a low frequency in the IPSS high risk category **(Table 1)**[3-5]. *TET2* mutations have been found in patients with other malignancies apart from MDS **(Table 2) (chapter 4).** Thus finding a *TET2* mutation is not specific for MDS, but could contribute to its diagnosis, especially in the IPSS low and intermediate risk groups.

Table 1: *TET2 mutations in myelodysplastic syndromes*

Reference	No. of patients	*TET2* mutations overall	*TET2* mutations in IPSS subgroups			
			low	int-1	int-2	high
This thesis	102	26%	12/29 (41%)	10/35 (29%)	3/24 (13%)	2/14 (14%)
2	115	23%	4/35 (11%)	13/39 (28%)	8/26 (31%)	1/15 (7%)
3	35	11%	1/11 (9%)	2/8 (25%)	0/4 (0%)	1/7 (14%)
4	59	22%	2/17 (12%)	8/23 (35%)	3/12 (25%)	0/7 (0%)
5	320	12%	ns	ns	ns	ns
1	439	21%	ns	ns	ns	ns

NS: Not specified

Table 2: *TET2 mutations in hematopoietic malignancies*

Hematopoietic malignancy	*TET2* mutations overall	Reference
Myeloproliferative neoplasms		
Chronic myeloid leukemia (only accelerated phase/blast crisis)	14-20%	65
polycythemia vera	10-16%	2, 6, 66, 67
primary myelofibrosis	8-27%	2, 6, 66, 67
essential thrombocythemia	2-10%	2, 6, 66, 67
mastocytosis	29%	68
Myeloid/lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB, FGFR1	absent	68
Myelodyplastic/Myeloproliferative neoplasms		
chronic myelomonocytic leukemia	20-50%	2, 5, 6, 69-74
juvenile myelomonocytic leukemia	absent	74-76
RARS-T*	9-26%	2, 74, 77, 78
Myelodysplastic syndromes	11-26%	***This thesis,*** 2-5, 74, 79 1
Acute myeloid leukemia	12-27%	***This thesis,*** 4, 6, 22, 23, 25, 79, 80
Blastic plasmacytoid dendritic cell neoplasm	53% *(in 13 patients)*	81
B-cell lymphoma	2%	18
T-cell lymphoma	12%	18
Acute lymphoblastic leukemia	absent	***This thesis***

** RARS-T is provisionally catagorized as a MDS/MPN*
"absent": tested in a limited number of patients so far, preventing a definitive conclusion concerning the presence of TET2 defects in these disorders

Tet proteins are involved in hydroxylation of 5-methylcytosine

The family of mammalian Tet proteins consists of Tet1, Tet2 and Tet3 **(Figure 1)**. Tet1, the founding member of the Tet proteins, was independently identified by several groups as a fusion partner of MLL in patients with acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). Mutations of the *TET1* and *TET3* genes have not been described[6]. The Tet proteins share two C-terminal highly conserved regions, which are also present in their orthologues in a variety of species. Tahiliani et al. discovered that these conserved regions are partly homologous to the predicted oxygenase (2OGFeDO) domain of the trypanosome proteins JBP1 and JBP2[7]. These proteins are involved in the hydroxylation of the methyl group of thymine in trypanosomes. Tet1 was shown to be involved in the hydroxylation of 5-methylcytosine (5-mC). Subsequent studies showed that Tet2 and Tet3 are involved in the same process[8, 9]. In order to execute their function, Tet proteins must bind iron (Fe^{2+}) and 2-oxoglutarate (or ketoglutarate). The functional consequences of 5-mC hydroxylation are an ongoing subject of studies. On the one hand, 5-mC hydroxylation may lead to passive demethylation. In this case, the methyltransferase DNMT1 no longer methylates its target cytosine when the complementary strand contains a 5-hydroxy-methylcytosine (5-hmC) instead of 5-mC. As a result, methylation patterns are no longer conserved in dividing cells. On the other hand, Tet proteins have been implicated in active demethylation. In this model, hydroxylation of 5mC is the first step in a number of modifications (**Figure 2**). After formation of 5-hmC, conversion to 5-carboxylcytosine (5-caC), 5-formylcytosine (5-fC) and 5-hydroxyuracil (5-hmU) may take place, leading, via interaction with various glycosylases and proteins of the base excision repair pathway to cytosine[10-12]. To date, not much is known about the presence of 5-hmC in healthy and malignant hematopoietic cells. Ko et al. showed that 5-hmC levels are reduced in those patients with myeloid malignancies, in whom *TET2* mutations were detected[9]. However, some patients in whom *TET2* was not mutated carried low 5-hmC levels as well, indicating that other proteins may affect the function of Tet2 or are involved in 5-mC hydroxylation themselves. Two of these proteins may be Isocitrate dehydrogenases (Idh) 1 and 2. Idh1 and Idh2 normally catalyze the conversion of isocitrate to 2-oxoglutarate. When mutated, they gain a novel function, namely the conversion of 2-oxoglutarate to 2-hydroxyglutarate (2-HG)[13, 14]. This may disrupt the function of 2-oxoglutarate-dependent proteins, such as Tet2. Considering the clinical heterogeneity of the diseases associated with *TET2* aberrations and the fact that *TET2* mutations co-occur with many other genetic defects, it seems unlikely that *TET2* mutations alone cause hematopoietic malignancies **(Tables 2** and **3).** Studies in *TET2* conditional knockout mouse models, have shown that loss of the *TET2* gene leads to expansion of the hematopoietic stem cell compartment and eventually a phenotype mostly resembling CMML [15-19]. In conclusion, mutations in the *TET2* gene are thought to lead to aberrant DNA methylation patterns, disturbing the expression of genes that influence cell differentiation and survival. Which genes are affected by these aberrant methylation patterns, and how this leads to the development of diverse malignancies, requires combined analysis of *Tet2*'s target genes and their expression levels. There is also little known about the differences in function between the Tet1, Tet2 and Tet3 proteins. The results of knock-down experiments suggest that these proteins are not mutually exclusive. If formation of 5-hmC physiologically leads to demethylation and if defects in the Tet proteins disturb this process, this is in agreement with the hypothesis that hypermethylation

contributes to development of myeloid malignancies. As described in the introduction of this thesis, demethylating agents are available in clinical practice and are currently under investigation in clinical trials. It is not known if patients with defects of Tet proteins show a specific response to these agents. The largest study, performed in 86 MDS patients (13 carrying a *TET2* mutation) treated with azacitidine, reported a better response rate in patients carrying a *TET2* mutation, but found no differences in response duration or overall survival[20].

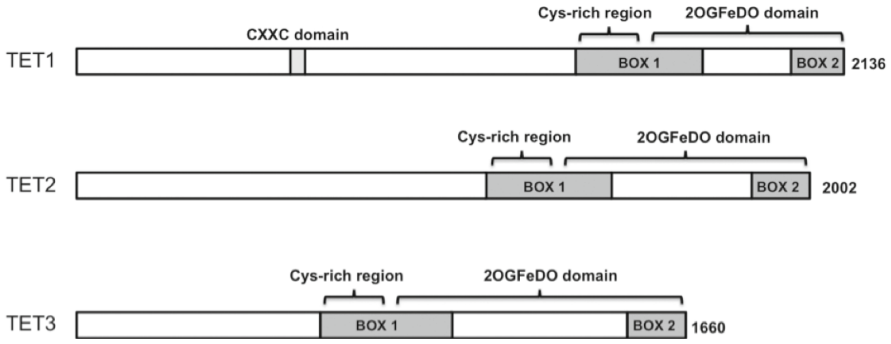


Figure 1: Tet family of proteins
Schematic representation of the three Tet proteins. Box 1 and Box 2 are conserved regions between the Tet orthologues and homologues and contain a Cys-rich region and the 2-oxoglutarate (2OG) and iron (Fe^{2+} binding domain. Tet1 is distinguished by the DNA binding CXXC domain.

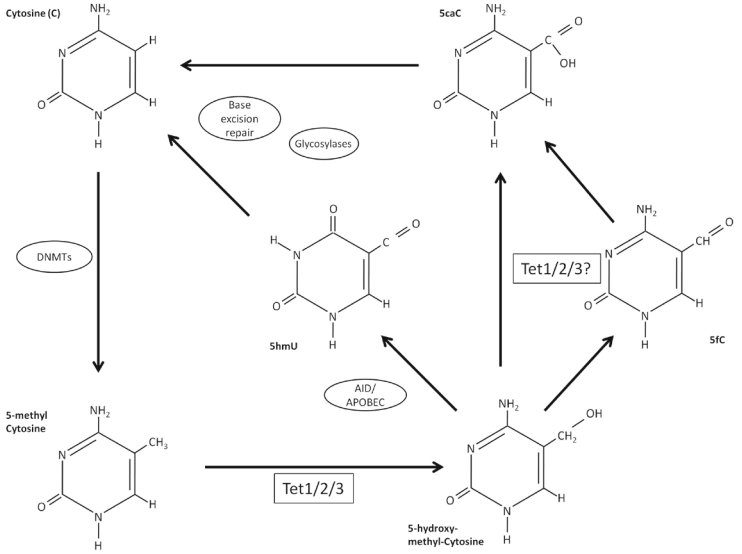


Figure 2: Role of the Tet proteins in active demethylation
Tet1, Tet2 and Tet3 are involved in the hydroxylation of 5-methylcytosine. When this process has taken place, 5-hydroxy-methylcytosine is thought to be converted to either 5-hydroxyuracil (5hmU), 5-carboxylcytosine (5caC) or 5-formylcytosine (5fC) and subsequently to cytosine. AID: activation-induced deaminase. APOBEC: apolipoprotein B mRNA-editing enzyme complex. DNMTs: DNA methyltransferases.

Tabel 3: Mutations co-occurring with TET2 mutations*

Gene	Function	Hematopoietic malignancy
JAK2	Signal transduction	MDS, MPN (PV, ET, PMF), MDS/MPN[1, 2, 5, 6, 25, 57, 66-68, 71, 74, 78, 82]
KIT	Signal transduction	MPN (systemic mastocytosis), (secondary) AML[68, 80, 83]
MPL	Signal transduction	MPN (PMF), MDS/MPN (RARS-T)[2, 6]
Flt3	Signal transduction	(secondary) AML, MDS/MPN (CMML)[14, 22, 23, 25, 80, 82, 83]
RAS	Signal transduction	MDS, MDS/MPN (CMML), (secondary) AML[1, 25, 57, 69-71, 80, 82, 83]
PTPN11	Signal Transduction	MDS/MPN (CMML)[70]
MLL	Histone methylation	AML[80]
EZH2	Histone methylation	MDS, MDS/MPN (CMML)[1, 57, 69, 82] ***(this thesis)***
UTX	Histone demethylation	MDS/MPN (CMML)[69]
DNMT3A	DNA methylation	MDS/MPN (CMML)[69, 80]
ASXL1	Chromatine remodelling	MDS, MPN, MDS/MPN, (secondary) AML[1, 4, 57, 69, 70, 72, 80, 82, 84]
c-Cbl	E3 ubiquitin ligase	MDS, MPN (MPN-U), MDS/MPN, (secondary) AML [1, 25, 57, 65, 69, 71]
NPM1	Nucleo-cytoplasmic transport	MDS, MDS/MPN (CMML), (secondary) AML [1, 4, 22, 23, 25, 57, 80, 83]
RUNX1	Transcription factor	MDS, MDS/MPN (CMML), (secondary) AML[1, 25, 57, 70, 71]
CEBPalpha	Transcription factor	(secondary) AML[14, 23, 25, 80]
WT1	Transcription factor	(secondary) AML[14, 23, 83]
PHF6	Regulation transcription	AML[14]
TP53	Apoptosis, DNA repair, cell cycle regulation	MDS, AML[1, 14]
IDH1/IDH2	Isocitrate dehydrogenase	MDS, MPN, MDS/MPN, (secondary) AML [23, 25, 69, 70, 85, 86]

**Of note, co-occurrence has been described in individual patients, in most cases it has not been proven that co-occurrence occurs in the same cell.*

Influence of TET2 aberrations on prognosis

In the MDS patient cohort described in this thesis, no significant difference in overall survival between *TET2* mutated and wild type patients was found. In the literature, there is no consensus regarding the impact of *TET2* mutations on prognosis. Kosmider et al. found a significantly better overall-, leukemia-free and event-free survival of *TET2* mutated MDS patients (n=88)[21]. When studying separate IPSS (international prognostic scoring system) groups, the overall survival of *TET2* mutated patients was significantly better than *TET2* wt patients in the IPSS low and int-1 risk groups, but not (significantly) in the int-2 and high risk populations. Another study by Smith et al. showed no significant difference in overall survival[5]. Interpretation of survival data in MDS is difficult due to the heterogeneity of the disease and of the treatment patients receive. We did not find a statistically significant correlation between *TET2* allelic burden and WHO classification or IPSS classification in MDS patients. However, it must be noticed that the sequencing technique we used is not sensitive enough to detect *TET2* mutations in small subpopulations. The effect of the *TET2* allelic burden may of course be influenced by the co-occurrence of other genetic defects. In two Tet2 knockout mouse models, it was shown that both homozygous and heterozygous deletions of the *TET2* gene resulted in a similar phenotype[16, 19]. A third study showed a more profound phenotype in the homozygous knockout[17].

In AML, studies performed so far mostly indicate a negative impact of *TET2* mutations on prognosis. Nibourel et al. reported no significant difference in overall and disease free survival in a cohort of 111 AML patients. However, their cohort contained only patients who achieved complete remission,

which may have influenced the outcome[22]. Metzeler et al. found an unfavorable prognostic impact of *TET2* mutations in the ELN (European Leukemia Network) favorable AML group (normal karyotype + CEBPa^{mut}/NPM1^{mut}, not Flt3^{mut}), but not in the remainder of their karyotypically normal AML cohort[23]. Chou et al. found a worse prognosis in patients with *TET2* mutations and intermediate risk cytogenetics[24]. In addition, Weissman et al. described a worse prognosis in AML patients with normal karyotypes if *TET2* mutations were present[25].

EZH2 mutations in malignant hematopoiesis

Although the role of Tet2 in epigenetics has only recently been partially elucidated, the function of Ezh2 has been the subject of various studies prior to the detection of mutations in hematological malignancies. The SET-domain of Ezh2 possesses methyltransferase activity which enables it to add methyl groups to lysines in histones. In case of Ezh2, histone 3 lysine 27 (H3K27) is its main substrate. H3K27 can be mono-, di- or trimethylated. Trimethylation of H3K27 is associated with a more compact chromatin structure and ultimately gene silencing. Ezh2 cannot perform this activity by itself, but needs to be incorporated in the polycomb repressive complex 2 (PRC2)[26-29](**Figure 3**). Mutations in other components of the PRC2, SUZ12, and EED, have been described in a small number of MDS/MPN patients[30]. Additional proteins have been described that are not part of PRC2 itself, but are able to modulate its activity. For example, Jarid2, belonging to the jumonji family of proteins, interacts with Ezh2. The target genes of Jarid2 and PRC2 show a large degree of overlap. *JARID2* was one of the genes which we found to be deleted in multiple patients using SNP-array analysis (**Chapter 3**). Point mutations of the *JARID2* gene have not been found in our patient cohort [31], but they have been described in 2% of MDS/MPN patients[30]. Ezh2 expression patterns have been studied in various cell populations during normal hematopoiesis. In addition to a role of Ezh2 in early hematopoietic differentiation, it has been implicated in B- and T-cell development[32-37]. PRC2 mediated modification of histones is important to establish and maintain gene expression patterns during various stages of development, such as stem cell maintenance, lineage commitment and senescence. Accordingly, an increase or decrease in the activity of the PRC2 or changes in its substrate specificity may result in severe disruption of these processes. In normal and malignant hematopoietic cells, Ezh2 expression was shown to correlate with increased proliferation[38]. In the past, overexpression of Ezh2 has been linked to various malignancies, including carcinomas of the prostate, breast, stomach, liver, lung and bladder[39-43]. Overexpression of Ezh2 in these malignancies has been related to increased aggressiveness of the tumor and poor clinical outcome. Support for the hypothesis that Ezh2 was involved in oncogenesis was found in experiments in which knockdown of Ezh2 in cancer cells resulted in growth arrest[44, 45], diminished tumor growth[46] and metastasis[47], whereas ectopic overexpression induced growth factor independence[45].

The mechanisms leading to increased Ezh2 expression in malignancies have been partially elucidated. In a subset of cancers, the *EZH2* genomic locus is amplified[39, 40, 44, 48] directly leading to overexpression. Alternatively, expression of *EZH2* is disturbed by defects in *EZH2* regulating

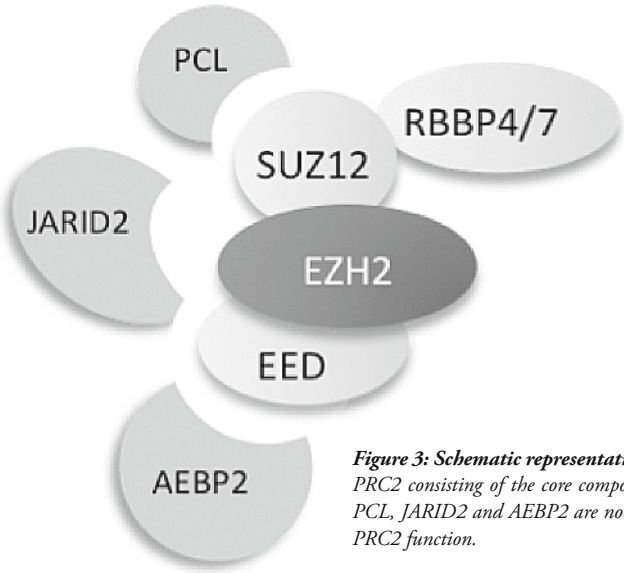


Figure 3: Schematic representation of the Polycomb repressive complex 2
PRC2 consisting of the core components EZH2, SUZ12, EED and RBB4/7. PCL, JARID2 and AEBP2 are not part of PRC2 but are thought to influence PRC2 function.

factors. For example, micro-RNA 101 has been shown to inhibit *EZH2*. In a subset of prostate and bladder carcinoma cells, levels of micro-RNA 101 were found to be reduced, either by somatic loss of the genomic locus or by other mechanisms, leading to increased *EZH2* levels[49, 50]. In addition, molecular pathways, such as those involving pRB-E2F and P53, have been shown to suppress *EZH2* expression, an activity that might be lost in cancer where these pathways are frequently found to be disturbed[39, 51].

In 2010, mutations of the *EZH2* gene were reported in follicular and diffuse large B-cell lymphomas[52]. The mutations all resulted in replacement of a tyrosine on position 641 in the SET domain of Ezh2 (Y641). In vitro assays measuring trimethylation activity of four different mutants showed that the ability of the mutants to trimethylate the H3K27 peptide was markedly reduced. Thus, it appeared that loss of Ezh2 function contributed to the pathogenesis of these malignancies, in contrast to the Ezh2 overexpression reported in many other types of cancer. However, subsequent studies have reported a decreased ability of the Ezh2 Y641 mutants to trimethylate unmethylated or monomethylated substrates but an increased ability to trimethylate dimethylated H3K27. As long as a wild type copy of Ezh2 is present, it compensates for the mutant's decreased affinity for unmethylated and monomethylated substrates. Overall, this results in an increase of H3K27Me3, in line with the expected increase in H3K27Me3 in cells overexpressing Ezh2[53, 54].

We detected various mutations located in different regions of the *EZH2* gene in MDS. Although part of the mutations are missense mutations, which may result in a similar change of substrate specificity of Ezh2 as reported for the Y641 mutation, nonsense mutations predicted to lead to a truncated protein were identified as well. Since all predicted truncated proteins lack (part of) the SET domain, which confers Ezh2's catalytic activity, these mutations most likely lead to loss of function. Several other groups have reported similar mutations in myeloid malignancies (**Table 4**). These findings are in contrast with the reports of increased Ezh2 activity in diverse malignancies. Although studies have

thus far shown that Ezh2 depletion leads to decreased proliferation and tumor growth, in specific circumstances, reduced expression of Ezh2 may contribute to leukemogenesis. For example, it has been shown that in hematopoietic stem cells, several genomic regions carry both active (H3K4me2 and H3K4me3) and repressive (H3K27me3) histone marks[55]. During differentiation, the balance between these marks shifts, favoring either activation or repression of specific genes. In some cases the bivalent pattern persists during differentiation. A shift in this balance by defects in the *EZH2* gene, may lead to increased expression of genes involved in oncogenesis.

Table 4: EZH2 mutations in hematopoietic malignancies

Hematopoietic malignancy	EZH2 mutations overall	Reference
Myeloproliferative neoplasms		
chronic myelogenous leukemia (only atypical and accelerated phase/blast crisis)	0-13%	56
polycythemia vera	3%	56
primary myelofibrosis	6-13%	56, 82, 87
essential thrombocythemia	absent	56
mastocytosis	absent	56
Myeloid/lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB, FGFR1	absent	56
Myelodyplastic/Myeloproliferative neoplasms		
chronic myelomonocytic leukemia	11-13%	56, 57, 82
Myelodysplastic syndromes	6%	***This thesis,*** 1, 56
Acute myeloid leukemia	absent	56 6, 79
Lymphomas		
Immunodeficiency-related NHL (exon 15) (2xTyr641, 1x other locus within SET domain)	3%	88
follicular lymphoma (Tyr641)	7-12%	52, 89
splenic B-cell mariginal zone lymphoma (entire gene)	absent	90
diffuse large B-cell lymphoma (Tyr641)	10%	52
mantle cell lymphoma, small lymphocytic lymphoma, peripheral T-cell lymphoma (Tyr641)	absent	52

Influence of EZH2 aberrations on prognosis

The *EZH2* gene is located on the long arm of chromosome 7. Deletions of the long arm of chromosome 7 have been associated with a worse prognosis in MDS patients. In our patient cohort, we also observed that overall survival in this group was reduced. The contribution of the deletion

of the *EZH2* gene to this phenomenon is unknown. However, patients with an *EZH2* mutation without a 7(q) deletion appeared to have a worse prognosis as well, although this must be interpreted with caution, since we did not study this effect in a prospective study of a homogeneously treated patient population. Similar results, pointing towards a worse prognosis in MDS and MDS/MPN patients carrying *EZH2* mutations, have been found by others[1, 56, 57].

TET2 and *EZH2*

The detection of mutations in the *TET2* and *EZH2* genes implicates two novel genes, involved in epigenetics, in the pathogenesis of MDS. In our MDS patient population, co-occurrence of *TET2* and *EZH2* mutations was frequently detected, pointing towards defects in both DNA and histone methylation. Loss of *Tet2* protein function may lead to DNA hypermethylation, whereas the mutations found in *EZH2* are proposed to lead to decreased H3K27 trimethylation. This raises the question if and how these processes are related. It has been hypothesized that binding of the PRC2 complex to DNA leads to recruitment of DNA methyltransferases and subsequent de novo methylation[58-60] in cancer cells. This phenomenon might explain increased DNA methylation in case of Ezh2 overexpression. However, in MDS we assume that normal Ezh2 function is lost. In contrast to the hypothesis mentioned above, other studies have found that several well known targets of DNA hypermethylation, such as CDKN2A, show no H3K27 trimethylation, and, likewise, not all H3K27 trimethylated genes show DNA hypermethylation. Also, inactivation of Ezh2 leads to reactivation of genes silenced by H3K27 trimethylation, but not of genes silenced by DNA methylation[61-63]. In case of Tet1 it has been suggested that its binding to genomic targets in embryonic stem cells partly co-occurs with both activating and repressing (such as H3K27) histone modifications[64]. However, in genome wide studies it proves difficult to determine if all modifications occur on the same DNA strand. Processes involving DNA and histone methylation thus seem to be intertwined, but to what extent, under which circumstances and at which genomic regions remains to be elucidated. Combining techniques such as Chip-sequencing and RNA-sequencing may in the future resolve some of these questions.

Conclusion

This thesis describes the identification of genetic aberrations in patients suffering from MDS. Two novel defects, those in the *TET2* and *EZH2* genes are studied in more detail in patients with MDS and other hematopoietic malignancies. Both defects are thought to result in deregulation of the epigenetic processes of DNA and histone methylation. In what way this deregulation results in aberrant differentiation, proliferation and cell death remains to be elucidated.

Table 5: Mutations co-occurring with EZH2 mutations

Gene	Function	Hematopoietic malignancy
JAK2	Signal transduction	MPN, MDS/MPN[57, 87]
MPL	Signal transduction	MPN[87]
RAS	Signal transduction	MDS, MDS/MPN (CMML)[1, 57]
ASXL1	Chromatine remodelling	MPN, MDS/MPN (CMML)[57, 82]
TET2	5-mC hydroxylation	MDS, MDS/MPN (CMML)[57, 82], ***This thesis***
c-Cbl	E3 ubiquitin ligase	MDS, MDS/MPN (CMML)[1, 57]
NPM1	Nucleo-cytoplasmic transport	MDS[1]
RUNX1	Transcription factor	MDS, MDS/MPN (CMML)[1, 57]
ETV6	Transcription factor	MDS[1]
TP53	Apoptosis, DNA repair, cell cycle regulation	MDS[1]
IDH1/2	Isocitrate dehydrogenase	MDS, MDS/MPN (CMML)[1, 57]

**Of note, co-occurrence has been described in individual patients, in most cases it has not been proven that co-occurrence occurs in the same cell.*

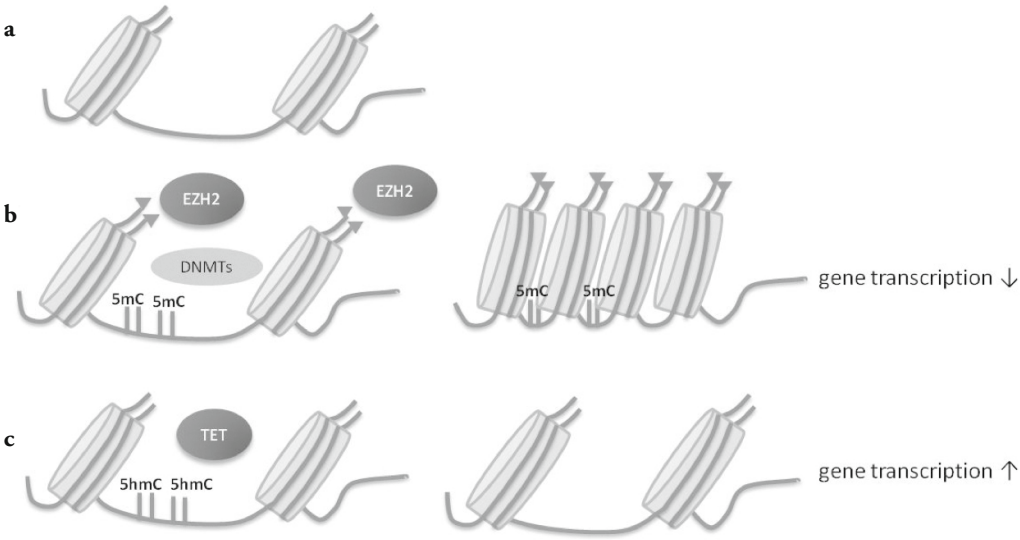


Figure 4: Possible role of EZH2 and TET2 in gene expression
An active configuration of chromatin, represented in (a) lacks trimethylation of histone H3K27 and contains unmethylated DNA. Trimethylation of H3K27 by EZH2 (as part of PRC2) and methylation of cytosines are thought to lead to a more condense chromatin structure and decreased gene expression (b). Cytosine methylation is thought to be reversed by a multistep process of which conversion of 5mC to 5hmC by Tet proteins is a first step. This could lead to increased gene expression (c).

Reference List

1. Bejar,R. et al. Clinical effect of point mutations in myelodysplastic syndromes. N. Engl. J. Med. 364, 2496-2506 (2011).
2. Delhommeau,F. et al. Mutation in TET2 in myeloid cancers. N. Engl. J. Med. 360, 2289-2301 (2009).
3. Gelsi-Boyer,V. et al. Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. Br. J. Haematol. 145, 788-800 (2009).
4. Rocquain,J. et al. Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. BMC. Cancer 10, 401 (2010).
5. Smith,A.E. et al. Next-generation sequencing of the TET2 gene in 355 MDS and CMML patients reveals low-abundance mutant clones with early origins, but indicates no definite prognostic value. Blood 116, 3923-3932 (2010).
6. bdel-Wahab,O. et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 114, 144-147 (2009).
7. Tahiliani,M. et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930-935 (2009).
8. Ito,S. et al. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature 466, 1129-1133 (2010).
9. Ko,M. et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468, 839-843 (2010).
10. He,Y.F. et al. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science 333, 1303-1307 (2011).
11. Ito,S. et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science 333, 1300-1303 (2011).
12. Guo,J.U., Su,Y., Zhong,C., Ming,G.L., & Song,H. Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. Cell 145, 423-434 (2011).
13. Xu,W. et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell 19, 17-30 (2011).
14. Figueroa,M.E. et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 18, 553-567 (2010).
15. Pronier,E. et al. Inhibition of TET2-mediated conversion of 5-methylcytosine to 5-hydroxymethylcytosine disturbs erythroid and granulomonocytic differentiation of human hematopoietic progenitors. Blood 118, 2551-2555 (2011).
16. Ko,M. et al. Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. Proc. Natl. Acad. Sci. U. S. A 108, 14566-14571 (2011).
17. Li,Z. et al. Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. Blood 118, 4509-4518 (2011).
18. Quivoron,C. et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell 20, 25-38 (2011).

19. Moran-Crusio,K. et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell 20, 11-24 (2011).

20. Itzykson,R. et al. Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. Leukemia 25, 1147-1152 (2011).

21. Kosmider,O. et al. TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDS). Blood(2009).

22. Nibourel,O. et al. Incidence and prognostic value of TET2 alterations in de novo acute myeloid leukemia achieving complete remission. Blood 116, 1132-1135 (2010).

23. Metzeler,K.H. et al. TET2 mutations improve the new European LeukemiaNet risk classification of acute myeloid leukemia: a Cancer and Leukemia Group B study. J. Clin. Oncol. 29, 1373-1381 (2011).

24. Chou,W.C. et al. TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. Blood 118, 3803-3810 (2011).

25. Weissmann,S. et al. Landscape of TET2 mutations in acute myeloid leukemia. Leukemia(2011).

26. Cao,R. et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298, 1039-1043 (2002).

27. Czermin,B. et al. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111, 185-196 (2002).

28. Kuzmichev,A., Nishioka,K., Erdjument-Bromage,H., Tempst,P., & Reinberg,D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. 16, 2893-2905 (2002).

29. Muller,J. et al. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111, 197-208 (2002).

30. Score,J. et al. Inactivation of polycomb repressive complex 2 components in myeloproliferative and myelodysplastic/myeloproliferative neoplasms. Blood(2011).

31. Kroeze,L.I. et al. Genetic defects in PRC2 components other than EZH2 are not common in myeloid malignancies. Blood 119, 1318-1319 (2012).

32. Kamminga,L.M. et al. The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. Blood 107, 2170-2179 (2006).

33. Lessard,J., Baban,S., & Sauvageau,G. Stage-specific expression of polycomb group genes in human bone marrow cells. Blood 91, 1216-1224 (1998).

34. Su,I.H. et al. Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. Nat. Immunol. 4, 124-131 (2003).

35. Su,I.H. et al. Polycomb group protein ezh2 controls actin polymerization and cell signaling. Cell 121, 425-436 (2005).

36. Koyanagi,M. et al. EZH2 and histone 3 trimethyl lysine 27 associated with Il4 and Il13 gene silencing in Th1 cells. J. Biol. Chem. 280, 31470-31477 (2005).

37. Kheradmand,K.S. et al. EZH2-dependent chromatin looping controls INK4a and INK4b, but not ARF, during human progenitor cell differentiation and cellular senescence. Epigenetics. Chromatin. 2, 16 (2009).

38. Visser,H.P. et al. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br. J. Haematol. 112, 950-958 (2001).

39. Bracken,A.P. et al. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J. 22, 5323-5335 (2003).

40. Kleer,C.G. et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc. Natl. Acad. Sci. U. S. A 100, 11606-11611 (2003).

41. Bachmann,I.M. et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J. Clin. Oncol. 24, 268-273 (2006).

42. Weikert,S. et al. Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. Int. J. Mol. Med. 16, 349-353 (2005).

43. Matsukawa,Y. et al. Expression of the enhancer of zeste homolog 2 is correlated with poor prognosis in human gastric cancer. Cancer Sci. 97, 484-491 (2006).

44. Varambally,S. et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419, 624-629 (2002).

45. Croonquist,P.A. & Van,N.B. The polycomb group protein enhancer of zeste homolog 2 (EZH 2) is an oncogene that influences myeloma cell growth and the mutant ras phenotype. Oncogene 24, 6269-6280 (2005).

46. Yu,J. et al. Integrative genomics analysis reveals silencing of beta-adrenergic signaling by polycomb in prostate cancer. Cancer Cell 12, 419-431 (2007).

47. Takeshita,F. et al. Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo. Proc. Natl. Acad. Sci. U. S. A 102, 12177-12182 (2005).

48. Collett,K. et al. Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin. Cancer Res. 12, 1168-1174 (2006).

49. Varambally,S. et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science 322, 1695-1699 (2008).

50. Friedman,J.M. et al. The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. Cancer Res. 69, 2623-2629 (2009).

51. Tang,X. et al. Activated p53 suppresses the histone methyltransferase EZH2 gene. Oncogene 23, 5759-5769 (2004).

52. Morin,R.D. et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat. Genet. 42, 181-185 (2010).

53. Yap,D.B. et al. Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. Blood 117, 2451-2459 (2011).

54. Sneeringer,C.J. et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. Proc. Natl. Acad. Sci. U. S. A 107, 20980-20985 (2010).

55. Cui,K. et al. Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. Cell Stem Cell 4, 80-93 (2009).

56. Ernst,T. et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat. Genet. 42, 722-726 (2010).

57. Grossmann,V. et al. Molecular profiling of chronic myelomonocytic leukemia reveals diverse mutations in >80% of patients with TET2 and EZH2 being of high prognostic relevance. Leukemia 25, 877-879 (2011).

58. Schlesinger,Y. et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat. Genet. 39, 232-236 (2007).

59. Vire,E. et al. The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439, 871-874 (2006).

60. Ohm,J.E. et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat. Genet. 39, 237-242 (2007).

61. Kondo,Y. et al. Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat. Genet. 40, 741-750 (2008).

62. McGarvey,K.M., Greene,E., Fahrner,J.A., Jenuwein,T., & Baylin,S.B. DNA methylation and complete transcriptional silencing of cancer genes persist after depletion of EZH2. Cancer Res. 67, 5097-5102 (2007).

63. McCabe,M.T., Brandes,J.C., & Vertino,P.M. Cancer DNA methylation: molecular mechanisms and clinical implications. Clin. Cancer Res. 15, 3927-3937 (2009).

64. Williams,K. et al. TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 473, 343-348 (2011).

65. Makishima,H. et al. CBL, CBLB, TET2, ASXL1, and IDH1/2 mutations and additional chromosomal aberrations constitute molecular events in chronic myelogenous leukemia. Blood 117, e198-e206 (2011).

66. Hussein,K. et al. Cytogenetic correlates of TET2 mutations in 199 patients with myeloproliferative neoplasms. Am. J. Hematol. 85, 81-83 (2010).

67. Tefferi,A. et al. TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23, 905-911 (2009).

68. Tefferi,A. et al. Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia 23, 900-904 (2009).

69. Jankowska,A.M. et al. Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: UTX, EZH2, and DNMT3A. Blood 118, 3932-3941 (2011).

70. Gelsi-Boyer,V. et al. ASXL1 mutation is associated with poor prognosis and acute transformation in chronic myelomonocytic leukaemia. Br. J. Haematol. 151, 365-375 (2010).

71. Kohlmann,A. et al. Next-generation sequencing technology reveals a characteristic pattern of molecular mutations in 72.8% of chronic myelomonocytic leukemia by detecting frequent alterations in TET2, CBL, RAS, and RUNX1. J. Clin. Oncol. 28, 3858-3865 (2010).

72. Kosmider,O. et al. TET2 gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia. Haematologica 94, 1676-1681 (2009).

73. Tefferi,A. et al. Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia 23, 1343-1345 (2009).

74. Jankowska,A.M. et al. Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. Blood 113, 6403-6410 (2009).

75. Perez,B. et al. Genetic typing of CBL, ASXL1, RUNX1, TET2 and JAK2 in juvenile myelomonocytic leukaemia reveals a genetic profile distinct from chronic myelomonocytic leukaemia. Br. J. Haematol. 151, 460-468 (2010).

76. Muramatsu,H. et al. Mutations of an E3 ubiquitin ligase c-Cbl but not TET2 mutations are pathogenic in juvenile myelomonocytic leukemia. Blood 115, 1969-1975 (2010).

77. Szpurka,H. et al. Spectrum of mutations in RARS-T patients includes TET2 and ASXL1 mutations. Leuk. Res. 34, 969-973 (2010).

78. Flach,J. et al. Mutations of JAK2 and TET2, but not CBL are detectable in a high portion of patients with refractory anemia with ring sideroblasts and thrombocytosis. Haematologica 95, 518-519 (2010).

79. Langemeijer,S.M. et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat. Genet. 41, 838-842 (2009).

80. Shen,Y. et al. Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. Blood 118, 5593-5603 (2011).

81. Jardin,F. et al. TET2 and TP53 mutations are frequently observed in blastic plasmacytoid dendritic cell neoplasm. Br. J. Haematol. 153, 413-416 (2011).

82. bdel-Wahab,O. et al. Concomitant analysis of EZH2 and ASXL1 mutations in myelofibrosis, chronic myelomonocytic leukemia and blast-phase myeloproliferative neoplasms. Leukemia 25, 1200-1202 (2011).

83. Kosmider,O. et al. TET2 mutations in secondary acute myeloid leukemias: a French retrospective study. Haematologica 96, 1059-1063 (2011).

84. bdel-Wahab,O. et al. Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. Cancer Res. 70, 447-452 (2010).

85. Tefferi,A. et al. IDH1 and IDH2 mutation studies in 1473 patients with chronic-, fibrotic- or blast-phase essential thrombocythemia, polycythemia vera or myelofibrosis. Leukemia 24, 1302-1309 (2010).

86. Kosmider,O. et al. Mutations of IDH1 and IDH2 genes in early and accelerated phases of myelodysplastic syndromes and MDS/myeloproliferative neoplasms. Leukemia 24, 1094-1096 (2010).

87. Guglielmelli,P. et al. EZH2 mutational status predicts poor survival in myelofibrosis. Blood 118, 5227-5234 (2011).

88. Capello,D. et al. Mutations of CD79A, CD79B and EZH2 genes in immunodeficiency-related non-Hodgkin lymphomas. Br. J. Haematol. 152, 777-780 (2011).

89. Bodor,C. et al. EZH2 Y641 mutations in follicular lymphoma. Leukemia 25, 726-729 (2011).

90. Salido,M. et al. Absence of mutations of the histone methyltransferase gene EZH2 in splenic b-cell marginal zone lymphoma. Leuk. Res. 35, e23-e24 (2011).

Chapter 7

Nederlandse samenvatting

Dankwoord

Curriculum vitae

List of publications

Nederlandse samenvatting

Het menselijk lichaam kan niet zonder goed functionerende bloedcellen. Er zijn drie soorten bloedcellen te onderscheiden: rode bloedcellen (erythrocyten), witte bloedcellen (leukocyten) en bloedplaatjes (trombocyten). De rode bloedcellen zijn nodig voor het zuurstof transport naar alle organen, zoals hart en hersenen. Personen met een tekort aan goed werkende rode bloedcellen hebben bloedarmoede (anemie). De witte bloedcellen zijn nodig voor de afweer tegen bacteriën, virussen, schimmels en parasieten. Functioneren deze cellen niet goed, dan heeft een persoon een vergrote kans op (ernstige verlopende) infecties. De bloedplaatjes zorgen voor de stolling van bloed. Dus, bij een tekort hieraan krijgen mensen eerder blauwe plekken, neusbloedingen en blijven wonden langer en heftiger nabloeden. De verschillende soorten bloedcellen worden gemaakt in het beenmerg en ontstaan daar uit dezelfde voorloper cel, of stamcel. Bij mensen die lijden aan een myelodysplastisch syndroom (MDS) verloopt de ontwikkeling van stamcel naar rijpe bloedcellen niet naar behoren. Hierdoor ontstaan tekorten aan rijpe bloedcellen en functioneren de gevormde bloedcellen niet adequaat. Sommige personen met MDS ontwikkelen na verloop van tijd leukemie. Wat er precies ten grondslag ligt aan deze abnormale bloedcelvorming is niet goed bekend. Dit is het onderwerp van het onderzoek beschreven in dit proefschrift.

Bij een groot deel van de personen met MDS is in het verleden aangetoond dat er weliswaar rode en witte bloedcellen en bloedplaatjes worden aangemaakt in het beenmerg, maar dat veel van deze cellen sterven voordat ze het bloed bereiken. In **hoofdstuk 2** van dit proefschrift wordt onderzocht of dit komt door abnormale activiteit van genen die betrokken zijn bij apoptose. Genen zijn stukjes van het DNA die de code vormen voor eiwitten. Eiwitten op hun beurt zijn verantwoordelijk voor de uitvoering van alle processen die zich in een cel afspelen, zoals het stimuleren van celgroei of juist het remmen hiervan. Apoptose wordt ook geprogrammeerde celdood genoemd. Hiermee wordt bedoeld dat een cascade van reacties in de cel in gang wordt gezet, onder controle van verschillende factoren, die er uiteindelijk toe leidt dat een cel sterft. In voorgaand werk door andere onderzoekers wordt gesuggereerd dat apoptose gestoord is bij MDS, waardoor cellen sneller sterven. Dit heeft geleid tot het onderzoek dat is beschreven in hoofdstuk 2, waarin de activiteit van verschillende genen, zowel genen die apoptose bevorderen als remmen, gemeten is. Bij deze experimenten zijn beenmergcellen van patiënten met MDS vergeleken met die van gezonde mensen. Hieruit bleek dat er, op basis van de activiteit van alle genen samen, geen duidelijk onderscheid te maken is tussen personen met en zonder MDS. Wel konden enkele specifieke genen, zoals het gen *BIK*, onderscheiden worden waarvan de activiteit verschilde tussen de onderzochte groepen.
Een verstoorde activiteit van genen, zoals de apoptose-genen, kan bijdragen aan het ontstaan van MDS. Echter, de vraag blijft waardoor deze verstoring veroorzaakt wordt. Om hier meer inzicht in te krijgen, richt het onderzoek in **hoofdstuk 3** van dit proefschrift zich niet op de activiteit van genen, maar op de genen zelf. Zoals beschreven zijn genen stukjes van het DNA. DNA is een code, door wetenschappers weergegeven door vier verschillende letters: A, T, G en C. Het DNA van de

mens bestaat uit miljoenen A's, T's, G's en C's. Tezamen vormen zij de blauwdruk voor het lichaam. Het DNA is georganiseerd in 46 chromosomen. De chromosomen worden aangeduid met nummers (1 tot en met 22, van elke chromosoom twee kopieën) en, in het geval van geslachtschromosomen, met letters (X en Y bij mannen, X en X bij vrouwen). De helft van deze chromosomen erft een persoon van zijn vader, de andere helft van zijn moeder. Als een cel in het lichaam zich deelt, moet het DNA behouden blijven. Dus, voorafgaand aan een celdeling, wordt het DNA verdubbeld. Vervolgens wordt het gelijk verdeeld over de twee nieuwe cellen die uit de deling voortkomen. Soms ontstaan er ongewild veranderingen in de DNA code van een cel. Een "C" wordt bijvoorbeeld veranderd in een "G". Dit noemen we een mutatie. Als zo'n verandering eenmaal ontstaan is, wordt hij bij deling van de cel doorgegeven aan de nieuwe cellen die uit de deling voortkomen. Dit is niet de enige verandering die kan optreden. Zo kunnen er bij het verdubbelen van het DNA en het verdelen van chromosomen tijdens een celdeling ook grotere stukken van de DNA code verloren gaan (een deletie) of juist teveel DNA in de nieuwe cel eindigen (duplicatie of amplificatie). Een bijzonder soort van afwijkingen dat tijdens de celdeling kan ontstaan, noemen we uniparentale disomie. Hierbij wordt (een deel van) een chromosoom dat oorspronkelijk afkomstig is van de vader vervangen door een kopie van (een deel van) het chromosoom van de moeder. Een cel bevat dan nog steeds in totaal twee kopieën van het betreffende chromosoom, maar omdat de chromosomen die van vader en moeder afkomstig zijn, nooit helemaal exact dezelfde ATGC code bevatten, kunnen ook hierdoor problemen ontstaan. In hoofdstuk 3 wordt de zoektocht beschreven naar DNA afwijkingen in de bloedcellen van personen die lijden aan MDS. Om deze DNA afwijkingen op het spoor te komen, is gebruik gemaakt van zogenaamde "SNP (Single Nucleotide Polymorphism)-arrays". Met deze techniek is het mogelijk om het DNA van een patiënt te vergelijken met het DNA van gezonde mensen. Zo kan men bepalen of er een stukje van het DNA van een persoon ontbreekt (een deletie) of juist teveel is (duplicatie of amplificatie). Ook kunnen gebieden met uniparentale disomie worden opgespoord. Mutaties, daarentegen, kunnen niet worden opgespoord met de SNP-arrays. De analyses met de SNP-arrays brachten een groot aantal DNA afwijkingen aan het licht in het beenmerg van personen met MDS (hoofdstuk 3, figuur 1). Door de afwijkingen van een groot aantal patiënten te vergelijken, kan een schatting worden gemaakt over het belang van de afwijking. Als een afwijking bij veel patiënten voorkomt, is de kans groot dat deze betrokken is bij het ontstaan van de ziekte. Het vervolg van het onderzoek in hoofdstuk 3 heeft zich geconcentreerd op een van deze vaker voorkomende afwijkingen, namelijk een afwijking van chromosoom nummer 4. In de beenmergcellen van twee personen bleek een stuk van een van de twee chromosomen nummer 4 te ontbreken. Een vergelijkbaar gebied was bij vier andere personen betrokken bij uniparentale disomie. Bij het vergelijken van alle afwijkende gebieden op chromosoom 4, bleek er een klein stukje DNA bij al deze patiënten aangedaan te zijn: het stuk dat het *TET2* gen bevat. Vervolgens is onderzocht bij alle patiënten in de studie, dus ook degenen zonder grote, met de SNP-array zichtbare, afwijkingen op chromosoom 4, of er mutaties in het *TET2* gen opgetreden waren. Dit bleek inderdaad het geval. In totaal was bij 26% van de patiënten het *TET2* gen afwijkend. Hoewel dit misschien niet veel lijkt, is er niet eerder een gen beschreven dat bij zoveel MDS patiënten is aangedaan.

Het *TET2* gen bleek actief te zijn in verschillende soorten bloedcellen, waaronder twee soorten witte bloedcellen, granulocyten en B-cellen. Om te onderzoeken of het *TET2* gen ook gestoord is bij andere aandoeningen dan MDS, zijn daarom twee andere ziektebeelden onderzocht, waarbij de vorming van (onder andere) granulocyten en B-cellen onderdrukt is, namelijk acute myeloide leukemie (AML) en acute lymfatische leukemie (ALL). Omdat het laatst genoemde ziektebeeld relatief vaak bij kinderen voorkomt, is eerst het *TET2* gen onderzocht in het beenmerg van kinderen met AML of ALL. Dit wordt beschreven in **hoofdstuk 4**. Bij 4% van de kinderen met AML werden afwijkingen in het *TET2* gen gevonden. Bij de kinderen met ALL konden geen *TET2* veranderingen worden aangetoond. Ook in AML blijkt het *TET2* gen dus gestoord te zijn, hoewel bij een kleiner deel van de patiënten in vergelijking met MDS.

Mede naar aanleiding van de door ons en anderen beschreven mutaties, heeft ook de functie van het TET2 eiwit veel aandacht gekregen. Hoewel de DNA volgorde (de A, T, C en G) de opbouw van de eiwitten bepaalt, kan er in het DNA molecuul ook nog een code worden gestopt die mede bepaalt of dat stuk van het DNA wel of niet wordt afgelezen. Deze code bepaalt dus mede of genen op dit stuk DNA actief zijn. Andere onderzoekers hebben laten zien dat het TET2 eiwit is betrokken bij het chemisch veranderen van het DNA molecuul. Hoewel het nog niet helemaal duidelijk is, lijkt deze verandering betrokken te zijn bij het opnieuw leesbaar maken van DNA dat door andere eiwitten was afgesloten. Bij afwijkingen in het TET2 gen, kan het TET2 eiwit niet meer normaal functioneren. Hierdoor kan de leesbaarheid van DNA en dus de activiteit van vele andere genen mogelijk beïnvloed worden.

In **hoofdstuk 5** tenslotte wordt een andere afwijking belicht die gevonden is in het DNA van MDS patiënten met behulp van de SNP-arrays. Het betreft een afwijking op chromosoom 7. Afwijkingen op een deel van chromosoom 7 zijn in het verleden vaker gevonden bij personen met MDS. Deze personen blijken vaker een slechtere prognose te hebben dan degenen zonder deze afwijkingen. Het was echter niet bekend in welk gen of welke genen op chromosoom 7 zich afwijkingen bevonden die betrokken kunnen zijn bij het ontstaan van MDS. Door opnieuw de SNP-array resultaten van alle patiënten met een afwijking op chromosoom 7 te vergelijken, is het gelukt om een klein gebied op te sporen dat bij al deze patiënten overlapt. Dit gebied bevat het *EZH2* gen. Het eiwit waar dit gen voor codeert is, net als TET2, betrokken bij het reguleren van de leesbaarheid van het DNA. Alleen EZH2 doet dit op een andere manier. Het DNA zit strak ingepakt in eiwitten, genaamd histonen, en is daarmee niet altijd even bereikbaar voor andere eiwitten die genen kunnen activeren. EZH2 zorgt voor een chemische verandering van een van de histonen, waardoor de manier waarop het DNA is ingepakt verandert. Het uitschakelen van EZH2, bijvoorbeeld door de mutaties die wij hebben gevonden, heeft grote gevolgen voor dit proces. Hoewel dat niet in onze studie is aangetoond, is de verwachting dat hierdoor ook de activiteit van andere genen in de cel veranderd kan zijn.

Samengevat laat dit proefschrift zien dat er bij patiënten met MDS frequent veranderingen voorkomen in de *TET2* en *EZH2* genen en dat hierdoor mogelijk de activiteit van andere genen in de bloedcellen verandert, wat bij kan dragen aan het ontstaan van MDS en AML. Een van de groepen genen, waarvan een veranderde activiteit beschreven is bij MDS zijn de apoptose genen. Of veranderingen in apoptose genen ontstaan door afwijkingen in *EZH2* en *TET2* zal nader moeten worden onderzocht.

Dankwoord

Vele mensen hebben een bijdrage geleverd aan het tot stand komen van dit proefschrift en nu we op de laatste pagina's zijn aangekomen, wil ik hen dan ook bedanken.

Beste Theo, in 2001 spraken wij elkaar voor het eerst over de mogelijkheden van onderzoek binnen de hematologie. Jij regelde toen dat ik stage kon gaan lopen bij de groep van John Tisdale op het NIH. Toen ik in 2005 opnieuw langskwam, maar nu voor onderzoek in Nijmegen zelf, hielp je mij weer op weg. Ik heb veel geleerd van jouw kennis over myelodysplastische syndromen, je enthousiasme en inzet voor de kliniek en het laboratorium en het stimuleren van een nauwe samenwerking tussen beiden.

Beste Joop, de afgelopen jaren heb je een belangrijke rol gespeeld bij het begeleiden van het MDS onderzoek. Ik heb enerzijds kunnen profiteren van je praktische kennis over experimenten en je eigenschap om altijd de rode draad in het onderzoek te kunnen blijven zien, maar heb ook meer kunnen leren over 'de business' van het onderzoek doen, zoals het schrijven van beursaanvragen. Ik hoop dat je in de toekomst in de positie zult zijn om vele AIO's op deze manier te kunnen begeleiden.

Beste Bert, vanaf de eerste werkbesprekingen heb je altijd actief meegedacht over de mogelijkheden binnen het MDS onderzoek. Je volgde alle literatuur op de voet en je kwam dan ook regelmatig met een artikel langslopen. Ik hoop dat er nooit iets van dat enthousiasme verloren gaat.

Beste Roland, de SNP-arrays spelen een belangrijke rol in dit proefschrift en vanaf het begin ben jij hierbij betrokken geweest. Ook heb je de studie naar *TET2* mutaties bij kinderen met leukemie begeleidt. Ik wil je bedanken voor je hulp en kritische blik bij het analyseren en interpreteren van de data.

Beste Ruth, bij elk onderzoek in dit proefschrift ben jij als analist betrokken geweest. Ik heb, net als vele andere AIO's en postdocs, gebruik kunnen maken van jouw laboratoriumervaring. Maar los van het praktische werk, heb ik zowel de leuke als de minder leuke aspecten van het doen van onderzoek met je kunnen bespreken. Jij bent de stabiele factor in een onderzoeksgroep die steeds van samenstelling wisselt. En die stabiele factor heb ik graag naast mij als paranimf op de dag van mijn promotie.

Beste Thessa, jij bent als student bij de TRIAD/GFI groep begonnen, maar gelukkig daarna naar de TET groep overgestapt. Jouw manier van werken is een aanwinst voor het lab. Helaas is ons laatste 'klimuitje' al weer een tijd geleden, maar ik hoop dat we in de toekomst weer wat mooie routes kunnen uitzoeken. Ik vind het erg fijn dat je paranimf bij mijn promotie wilt zijn.

Beste Mariam, jij was mijn eerste student op het lab en in de tijd dat we de eerste TET2 mutatie vonden. We hadden toen nog geen idee wat dat gen deed, maar vonden het des te spannender om dit uit te gaan zoeken. Jij bent nu nog druk bezig om onderzoek te doen naar de functie van TET2. Dat is niet makkelijk gezien de toenemende concurrentie, maar ik weet zeker dat je je onderzoek tot een goed einde zal brengen.

Beste Gorica, er is volgens mij geen proef die je niet kan. Al je ervaring als analist stop je nu in je promotieonderzoek, dat ongetwijfeld een uitstekend proefschrift op zal leveren. Ik hoop dat het EZH2 artikel, waaraan we samen werkten, een mooie 'functionele' opvolger krijgt.

Beste dames en heer van de moleculaire diagnostiek. Eigenlijk vind ik dat de naam 'moleculaire diagnostiek' tekort doet aan de bijdrage die jullie leveren aan het onderzoek. Een groot deel van jullie heeft meegewerkt aan het onderzoek in dit proefschrift en jullie allemaal dragen bij aan de goede sfeer die ik altijd ervaren heb als ik weer eens eventjes binnen liep. Bedankt voor jullie hulp bij onder meer het sequencen van TET2 en/of EZH2. Patricia, ik zie ons nog zitten om met een lineaaltje de pieken te meten. Gelukkig zijn er meer betrouwbare technieken. Louis, dank voor alle hulp met de CARDs en het 'primer-en-probe' ontwerpen.

Beste Leonie, Sylvie en Maaike, het was heel fijn om op het lab met mensen zoals jullie samen te werken. Sylvie, je bent inmiddels naar Canada verhuist om je als post-doc op een nieuw onderzoek te storten. Als je dit met het doorzettingsvermogen doet waarmee je in de klimhal een route blijft proberen totdat je aan de top zit, komt het helemaal goed. Leonie, wij blijven hopelijk in de toekomst diezelfde klimroutes proberen. Dan zal ik het niet meer over Darwin hebben. Maaike, als jij jezelf een doel stelt, dan ga je daar volledig voor met een inzet waar ik alleen maar bewondering voor kan hebben. Je wordt ongetwijfeld een uitstekende kinderarts.

Beste Sas1 (of toch 2?), wij zijn kort na elkaar op het lab begonnen en inmiddels ben je een ervaren analiste. Er zijn niet veel mensen met wie ik 7 dagen en nachten vrolijk in de trein kan zitten, maar aan onze reis met de transmongolië-express heb ik alleen maar goede herinneringen.

Beste Aswin, Elke, Jeannet, Jurgen, Liesbeth en Sake, jullie werkten al een tijd op het lab toen ik ineens 'op de stoep stond'. Ik wil jullie bedanken voor alle gezelligheid tijdens het eerste deel van mijn AIO-tijd. Leilei, Jimmy en Davide, jullie zijn later op het lab gekomen en inmiddels zelf ook bezig met het laatste deel van het AIO-schap. Ik wens jullie veel succes bij het afronden van jullie proefschrift.

Beste Roderick, net als Mariam en Leonie heb jij als student meegewerkt aan het TET-project. Hoewel de resultaten van jouw inspanningen uiteindelijk niet in dit proefschrift staan, heb je me veel werk uit handen genomen tijdens je stage.

Beste Marieke, jij hebt je een jaar lang met de TET2 mutatie analyses bezig gehouden en hierdoor een belangrijke rol gespeeld bij het tot stand komen van het eerste TET2 artikel. Rob en Jeroen, bedankt voor het vele helpen met het sorteren van de beenmergcellen die voor de studies in dit proefschrift gebruikt zijn. Christian en Jan, bedankt voor de hulp met de statistische analyses. Eveline en Simon, bedankt voor de hulp met de SNP-arrays. Reinier, dank voor de kritische inbreng bij de werkbesprekingen.

Beste dames en heren van 'de morfologie', 'immuunfenotypering' en 'BMT-unit'; zonder de samenwerking met jullie was nooit de basis gelegd voor het onderzoek in dit proefschrift, namelijk het verzamelen en karakteriseren van patiëntenmateriaal.

Beste Peter, Estelle en Anne, dank voor de fijne samenwerking vanuit Leuven. De patiëntsamples met de nauwkeurig verzamelde klinische data hebben een belangrijke bijdrage geleverd aan het onderzoek in dit proefschrift.

Beste Marius en Petra, dankzij jullie is mijn beeld van 'de patiënt met MDS' niet beperkt gebleven tot het DNA. Jullie hebben mij, samen met Theo, de kans gegeven om op de polikliniek hematologie MDS patiënten te begeleiden binnen klinische studies. Daarnaast heb ik veel geleerd over de mogelijkheden en beperkingen van de diagnostiek naar MDS. Dit is een belangrijk onderdeel van mijn 'opvoeding' tot arts-onderzoeker.

Beste Fred, dank voor de snelle, uitvoerige en kritische beoordeling van mijn proefschrift.

Naast alle mensen met wie ik op het lab en in de kliniek heb samengewerkt, mogen nog een aantal anderen niet in dit dankwoord ontbreken.

Beste Carien, Lynn, Remco, Renate, Ruth, Surayda en Susan. Tijdens en na onze geneeskunde studie, hebben we vele leuke momenten gedeeld. Jullie hebben tijdens mijn onderzoek en nu specialisatie voor de nodige afleiding gezorgd.

Lieve papa en mama, ik wil jullie bedanken voor alle steun in de afgelopen jaren. Ik kan jullie zeggen dat ik heel wat 'correcties Langemeijer' heb moeten uitvoeren voorafgaand aan publicatie van dit proefschrift. Het is fijn om te weten altijd op jullie te kunnen rekenen.

Lieve Laurens, ik kan je nooit in een paar regels genoeg bedanken voor alles wat je voor me doet en voor me betekent. Het leven is zoveel mooier met jou.

Curriculum Vitae

Saskia Maria Carola Langemeijer werd op 4 juni 1980 geboren te 's Hertogenbosch. Na het behalen van het VWO diploma aan het Stedelijk Gymnasium in 's Hertogenbosch, begon ze in 1998 met de studie geneeskunde aan de Radboud Universiteit Nijmegen (toen Katholieke Universiteit Nijmegen). Van 2002 tot 2003 verruilde ze Nijmegen een klein jaar voor Washington DC om wetenschappelijk onderzoek te kunnen doen aan het National Institute of Diabetes Digestive and Kidney diseases in Maryland, USA. Deze stage vond plaats binnen de Molecular and Clinical Hematology branch onder leiding van dr J Tisdale. Eenmaal terug in Nederland behaalde ze in 2003 het doctoraal diploma (cum laude) en in 2005 het arts examen (cum laude). In 2005 startte ze haar promotie-onderzoek op het Laboratorium Hematologie van het UMC St Radboud onder leiding van dr J H Jansen. In 2007 kreeg zij een AGIKO stipendium en startte ze met de opleiding tot internist aan het UMC St Radboud (opleiders prof dr J.W.M van der Meer en later prof dr J de Graaf), waarna klinische stages en wetenschappelijk onderzoek elkaar afwisselden. Vanaf 2012 is ze internist in opleiding in het Canisius Wilhelmina Ziekenhuis te Nijmegen.

List of Publications

This thesis

- SMC Langemeijer, R Knops, C Gilissen, R Woestenenk, T de Witte, BA van der Reijden, JH Jansen. Apoptosis-related gene expression profiling in hematopoietic cell fractions of MDS patients. *Submitted*

- Langemeijer SM, Jansen JH, Hooijer J, van Hoogen P, Stevens-Linders E, Massop M, Waanders E, van Reijmersdal SV, Stevens-Kroef MJ, Zwaan CM, van den Heuvel-Eibrink MM, Sonneveld E, Hoogerbrugge PM, van Kessel AG, Kuiper RP. TET2 mutations in childhood leukemia. *Leukemia*. 2011 Jan;25(1):189-92.

- JH Jansen and SMC Langemeijer. The biological basis of MDS. *EHA education book* 2010

- SMC Langemeijer, MG Aslanyan, JH Jansen. TET mutaties komen frequent voor bij myeloide maligniteiten. *Nederlands tijdschrift voor Hematologie.* 2010;7:131-7

- Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tönnissen ER, vander Heijden A, Scheele TN, Vandenberghe P, de Witte T, van der Reijden BA, JansenJH. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet*. 2010 Aug;42(8):665-7.

- Langemeijer SM, Aslanyan MG, Jansen JH. TET proteins in malignant hematopoiesis. *Cell Cycle*. 2009 Dec 15;8(24):4044-8.

- Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, Stevens-Linders E, van Hoogen P, van Kessel AG, Raymakers RA, Kamping EJ, Verhoef GE, Verburgh E, Hagemeijer A, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH. Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nat Genet*. 2009 Jul;41(7):838-42.

- Langemeijer SM, de Graaf AO, Jansen JH. IAPs as therapeutic targets in haematological malignancies. *Expert Opin Ther Targets*. 2008 Aug;12(8):981-93.

Other publications

- Delnooz CC, Lefeber DJ, Langemeijer SM, Hoffjan S, Dekomien G, Zwarts MJ, Van Engelen BG, Wevers RA, Schelhaas HJ, van de Warrenburg BP. New cases of adult-onset Sandhoff disease with a cerebellar or lower motor neuron phenotype. *J Neurol Neurosurg Psychiatry*. 2010 Sep;81(9):968-72.

- Bosman G, Langemeijer SM, Hebeda KM, Raemaekers JM, Pickkers P, van der Velden WJ. The role of rituximab in a case of EBV-related lymphoproliferative disease presenting with haemophagocytosis. *Neth J Med*. 2009 Sep;67(8):364-5.

- Langemeijer SM, van Deuren M. A patient with redness and swelling of his foot: rheumatoid arthritis? *Neth J Med*. 2009 Jul-Aug;67(7):290-3.

- Hsieh MM, Langemeijer S, Wynter A, Phang OA, Kang EM, Tisdale JF. Low-dose parenteral busulfan provides an extended window for the infusion of hematopoietic stem cells in murine hosts. *Exp Hematol*. 2007 Sep;35(9):1415-20.

- Hussain SZ, Strom SC, Kirby MR, Burns S, Langemeijer S, Ueda T, Hsieh M, Tisdale JF. Side population cells derived from adult human liver generate hepatocyte-like cells in vitro. *Dig Dis Sci*. 2005 Oct;50(10):1755-63.

- Kang EM, Zickler PP, Burns S, Langemeijer SM, Brenner S, Phang OA, Patterson N, Harlan D, Tisdale JF. Hematopoietic stem cell transplantation prevents diabetes in NOD mice but does not contribute to significant islet cell regeneration once disease is established. *Exp Hematol*. 2005 Jun;33(6):699-705.

- Ueda T, Brenner S, Malech HL, Langemeijer SM, Perl S, Kirby M, Phang OA, Krouse AE, Donahue RE, Kang EM, Tisdale JF. Cloning and functional analysis of the rhesus macaque ABCG2 gene. Forced expression confers an SP phenotype among hematopoietic stem cell progeny in vivo. *J Biol Chem*. 2005 Jan 14;280(2):991-8.